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RESEARCH



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Director, Central Research Institute, Kasauli.

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Editor:—Lieut.-Colonel M. L. AHUJA, M.B., Ch.B., M.D., D.P.H.,
Director, Central Research Institute, Kasauli.

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AND OTHERS.

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Lieut.-Colonel K. R. K. IYENGAR, I.M.S. (*retd.*)
(1883-1947)

OBITUARY.

KOMBAR RAMASWAMY KRISHNASWAMY IYENGAR,

C.I.E., O.B.E., M.L.C., M.D., D.P.H.,

LIEUTENANT-COLONEL, I.M.S. (*retd.*).

It is with deep regret that we have to record the death on 20th July, 1947, of Lieut.-Colonel K. R. K. Iyengar, late Director of the Pasteur Institute of Southern India, Coonoor, following an attack of coronary thrombosis.

Colonel Iyengar received his medical training at the University of Edinburgh obtaining M.B., Ch.B. in 1908 followed by M.D. with honours and D.P.H. a few years later. He was selected for the Medical Research Department of the Government of India and was for about 14 years on the staff of the Central Research Institute, Kasauli. During this period he published a number of papers embodying his observations, chiefly on immunological problems. He was for many years one of the collaborators in the publication of this *Journal*. An experienced and capable research worker, he was elected President of the Medical Section of the Indian Science Congress in 1935 and awarded the Research Worker's Medal in 1939. He was Director of the Pasteur Institute, Coonoor, for 15 years and was responsible to a large extent for the success of the scheme of decentralization of antirabic treatment in the Madras Presidency. He joined the Indian Medical Service in 1921 and retired in 1938 but during World War II he volunteered for re-employment and served as Director of the Pasteur Institute, Coonoor, from 1941 to 1946.

His outstanding services met with well-merited recognition and he was awarded the O.B.E. in 1939 and C.I.E. in 1945. For his deep interest in social and political welfare of his country he was nominated

Member of the Legislative Council, Madras. His advice was often sought and highly valued both by the Government and the public.

His death is a serious loss to the country, for men so well equipped and experienced in professional and public affairs and so well qualified to command respect and affection of his countrymen are extremely rare. A charming friend, a wise councillor, a wonderful host and a great public speaker who, whenever called upon to address an audience, always did so in well-chosen and suitable language scintillating with refreshing wit and humour. In his younger days, he was a fine cricketer and a useful tennis player who collected many a trophy in local tournaments. He was the father of the Rotary Movement in the Nilgiris and was elected District Governor of the South India Rotary Clubs in 1943. He was one of those few great souls who really lived up to the Rotarian's motto : *Service above self*.

Colonel Iyengar was greatly interested in horticulture and, like everything that he undertook, he pursued it with great enthusiasm. A great lover of dogs and birds and a keen rose grower he made the Pasteur Institute garden one of the finest in the Nilgiris.

He had a wonderful knack of getting on with people of all sorts and types. His popularity was indicated by the presentation, subscribed for by the public, of an illuminated address and a large silver casket with the figure of the Pasteur Institute, Coonoor, embossed on it, given to him on retirement.

He married Usha Sinha, daughter of Lieut.-Colonel N. P. Sinha, I.M.S., elder brother of the first Lord Sinha. He leaves behind three daughters, two sons and a devoted wife who helped him in his many activities. Those who were privileged to know him and his family witnessed an affectionate devotion and understanding not easily forgotten. To the bereaved family we offer our sincere sympathy.

M. L. A.

Editor, I. J. M. R.

OBSERVATIONS ON CHOLERA VACCINE.

BY

M. L. AHUJA,

AND

GURKIRPAL SINGH.

(*From the Central Research Institute, Kasauli.*)

[Received for publication, May 20, 1947.]

PRESENTED below are the results of some of the laboratory investigations carried out in 1943-44 in human subjects and experimental animals on the assessment of immunity following prophylactic cholera vaccination :—

BACTERICIDAL POWER OF SERA OF HUMAN SUBJECTS AFTER INOCULATION WITH CHOLERA VACCINE.

Twenty-one male volunteers, 18 to 45 years of age, were inoculated with 1 c.c. of prophylactic cholera vaccine and their sera tested at various intervals for bactericidal power against homologous and heterologous subtypes of *V. cholerae*. The subjects were labourers who had never been previously inoculated with cholera vaccine. They belonged to Simla Hills States where there has been no epidemic of cholera for the past twenty years. None of them had ever lived in any endemic area. They were divided into three groups. One group received cholera vaccine prepared from *V. cholerae* subtype Inaba, the other group received cholera vaccine prepared from subtype Ogawa and the third group a mixed Inaba and Ogawa vaccines. The vaccines were prepared according to the method used at the Central Research Institute, Kasauli, i.e. by growing the organism on nutrient agar-papain digest of mutton—for 24 hours at 37°C., washing off the growth in sterile normal saline and killing it by the addition of 0.5 per cent phenol. Inaba *plus* Ogawa vaccine was prepared by pooling together equal parts of the two vibrio suspensions standardized to the same bacterial content, 8,000 million organisms per c.c. ★

The technique for the bactericidal test was a modification of Neisser and Wechsberg method. Briefly it consists of allowing culture, serum and complement to remain in contact for $4\frac{1}{2}$ hours at 37°C . Serial dilutions are then made from the mixture in sterile normal saline and a fixed inoculum sampled on nutrient agar plates or in 5 c.c. of nutrient broth. Evidence of survival or multiplication is shown by the number of viable bacteria as determined by colony counts on agar or by growth in broth. The latter being a more sensitive index was adopted as a routine in the tests carried out in this investigation.

The following technique has given reliable and consistent results in our hands and is described in detail.

TECHNIQUE.

Dilutions of serum.—A row of 13 tubes is numbered and 2 c.c. of sterile peptone water previously warmed to 37°C . is pipetted into each, with the exception of the first tube into which is run 2.7 c.c. of sterile peptone water. To tube I is added 0.3 c.c. of the serum under test. The contents are thoroughly mixed and 1 c.c. of the mixture carried over to tube II and so on, 1 c.c. being discarded from tube X. Tubes XI, XII and XIII do not receive immune serum but serve as controls (*vide infra*). The dilutions of serum thus prepared range from 1 in 10 to 1 in 100,000.

Complement-culture suspension.—A mixture in the proportion of 18 c.c. of fresh guinea-pig* serum and 2 c.c. of 18 hours' peptone-water culture of the vibrio subtype under test is prepared in a small sterile flask immediately before it is required to be added to the dilutions of the serum. This is well shaken before use.

By means of a sterile 2-c.c. pipette 1 c.c. of the mixture is added in turn to each tube except the last (tube XIII) which serves as the inoculum control and receives 1 c.c. of 18 hours' culture diluted with sterile peptone-water in the same proportion as the complement-culture suspension. Two other controls are put up in addition to the inoculum control for each test, the initial complement-culture control after $4\frac{1}{2}$ hours' incubation at 37°C . being represented by tube XI. Tube XII is sampled immediately after the addition of the complement-culture suspension to the various dilutions of serum.

Sampling.—The rack of tubes is placed in the incubator at 37°C . for $4\frac{1}{2}$ hours at the end of which period the contents of each of the 12 tubes are sampled in serial ten-fold dilutions in a range of 10^{-1} to 10^{-8} , 0.2 c.c. of each serial dilution being inoculated into 5 c.c. of broth for evidence of growth.

Separate pipettes must be used for each serial dilution and for each dilution of serum.

A sample protocol showing the results of tests is given below (Table I):—

* Not all guinea-pig sera are suitable for the purpose. Of 87 guinea-pigs tested 18 were found to have natural bacteriolysins and were considered unsuitable for yielding complement for this test.

TABLE I.

Human serum bactericidal titre 1 in 3,600.

Tubo No. :—	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	XI.	XII.	XIII.
Final dilution :—	15	45	135	405	1,215	3,630	10,890	33,000	100,000	300,000	Control		
Undiluted serum	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-1}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-2}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-3}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-4}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-5}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-6}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-7}	—	—	—	—	—	—	—	—	—	—	+	+	+
10^{-8}	—	—	—	—	—	—	—	—	—	—	+	+	+

XI. Complement-culture control after 4½ hours' incubation.

XII. Initial inoculum control sampled at the start of the test.

XIII. Inoculum control (same as XII) sampled after 4½ hours' incubation.
+ Growth.
— No growth.

Interpretation of results.—In the sample protocol above the initial inoculum control shows growth up to 10^{-4} . Allowing a margin of 1 tube above and one tube below 10^{-4} , any serum dilution which shows growth in 10^{-3} or less is considered as exerting bactericidal or bacteriostatic effect and any tube showing growth in 10^{-4} or over is taken as having no bactericidal effect. The results in this investigation have been interpreted on this basis.

THE RELATIVE VALUE OF INABA AND OGAWA STRAINS FOR PREPARATION OF CHOLERA VACCINE.

Bactericidal response in inoculated human subjects has been used as the test for assessing the immunological value of the strains.

Six volunteers were given a single dose of 1 c.c. of Inaba vaccine subcutaneously, six were similarly injected with 1 c.c. of Ogawa vaccine and six with a mixed vaccine containing equal parts of Inaba and Ogawa vaccines. Their sera were tested against homologous and heterologous subtypes of *V. cholerae*. The strains used for preparation of vaccines were different from those used for testing bactericidal power.

Samples of sera taken one day before inoculation of vaccine and tested for presence of natural bacteriolysins showed a maximum titre of 1 : 135 in 7, 1 : 45 in 3, and 1 : 15 or less in 8 subjects.

Bactericidal titres in the same subjects 10 days later were as follows: The reason for selecting the 10th day for doing comparative bactericidal tests was that experiments on the rate of development of immunity after vaccination (to be referred to later) showed that the maximum response was not developed till after the 8th day.

In order to determine if the rise in bactericidal power was specific against *V. cholerae*, samples of sera from six of the eighteen volunteers were tested, both before and after cholera vaccination, against (1) *B. typhosus*, (2) *B. coli* and three strains of N.A.G. vibrio isolated from non-cholera sources. There was no bactericidal effect against any of the strains tested.

(1) The results indicate that human subjects inoculated with cholera vaccine show a marked increase in bactericidal power rising from 0/135 before inoculation to anything between 400 and 100,000 ten days after vaccination.

(2) The development of bacteriolysins is specific for *V. cholerae*.

(3) Bacteriolysins are developed against both homologous and heterologous subtypes of *V. cholerae*.

(4) Bactericidal titres in subjects inoculated with Inaba type vaccine are somewhat higher than those inoculated with either Ogawa vaccine or a mixed Ogawa and Inaba vaccine.

(5) In the majority of cases the bactericidal titre against the homologous type is higher than against heterologous types.

TABLE II.

Bactericidal and agglutinin titres before and after cholera vaccination.

Subject number.	Strain used for vaccine.	PRE-VACCINATION TITRE.				POST-VACCINATION TITRE.			
		INABA.		OGAWA.		INABA.		OGAWA.	
		Vibrio- cidins.	Agglu- tinins.	Vibrio- cidins.	Agglu- tinins.	Vibrio- cidins.	Agglu- tinins.	Vibrio- cidins.	Agglu- tinins.
1	Inaba.	135	Nil 1/25	Nil	Nil 1/25	33,000	50	3,600	25
2		15	"	15	"	33,000	25/50	11,000	25
3		45	"	Nil	"	100,000	25/50	33,000	50/125
4		15	"	15	"	1,200	25	11,000	Nil
5		135	"	Nil	"	33,000	25	33,000	25
6		15	"	45	"	11,000	25/50	1,200	125/250
								,000	50/125
								,000	50/125
								,000	125
								,000	50
								,600	50/125
								,000	25/150
13	Inaba + Ogawa.	45	"	135	"	3,600	25	11,000	25/50
14		Nil	"	15	"	3,600	25/50	11,000	25/50
15		15	"	15	"	1,200	Nil	11,000	25/50
16		Nil	"	Nil	"	11,000	25/50	11,000	50/125
17		Nil	"	135	"	11,000	25/50	3,600	50/125
18		15	"	15	"	400	Nil	1,200	Nil

CORRIGENDUM.

On page 6, line 20, in the sentence 'Bactericidal titres in the same subjects 10 days later were as follows:', instead of 'as follows' read 'as given in Table II (see opposite)'.

Editor, I. J. M. R.

(6) In a number of cases the titre for both homologous and heterologous types is the same. In other words the bactericidal response evoked by cholera vaccine prepared from, say, an Inaba strain is not specific for Inaba types of vibrios only but is also effective against Ogawa strains and vice versa. The rise in bactericidal power is not related to the absence or presence of natural bacteriolysins before vaccination.

RATE OF DEVELOPMENT OF BACTERICIDAL POWER AFTER CHOLERA VACCINATION.

The development of bactericidal substances during the course of immunization and the relationship of these substances in determining the issue of experimental infection is described below.

Three subjects were inoculated with a single dose of 1 c.c. cholera vaccine prepared from an Inaba strain of *V. cholerae*. Their sera were tested six times during the first thirty days and once a month subsequently for the next five months. The rate of development of bactericidal power during the first six months is shown in Table III:—

TABLE III.

Production of bactericidins in man after a single injection of cholera vaccine.

Interval elapsing after vaccination, days.	VOLUNTEER 19.		VOLUNTEER 20.		VOLUNTEER 21.	
	Bactericidal titre.	Agglutination titre.	Bactericidal titre.	Agglutination titre.	Bactericidal titre.	Agglutination titre.
0	45	Nil	0	Nil	15	Nil
3	135	Nil	135	Nil	15	Nil
5	400	25/50	400	Nil	135	Nil
8	11,000	125/250	33,000	50/125	1,200	25/50
10	11,000	125/250	33,000	50/125	1,200	25/50
14	11,000	125	11,000	50/125	1,200	25
30	3,600	50	11,000	25/50	135	Nil
70	1,200	50	1,200	25	135	Nil
100	400	50	400	25	45	Nil
130	400	50	135	25	15	Nil
180	400	Nil	400	Nil	45	Nil

A gradual rise in bactericidal titre commencing on the 3rd day and attaining the maximum on the 8th day is shown in all the three subjects investigated. After the 8th day there is no further rise in bactericidal power and this remains more or less constant on the 10th and 14th days after vaccination. By the end of the 30th day the bactericidal titre shows a definite tendency to fall. As the period increases there is a gradual progressive lowering of the titre till by the 100th day the titre is less than a twentieth of the high level previously attained. By the end of six months the bactericidal power of the serum has reached its previous pre-inoculation level or is only slightly higher. Taking the development of bacteriolysins as

an indication of the development of immunity it can be assumed that up to the 3rd day there is practically no immunity, between the 3rd and 5th days there is partial immunity and after the 5th day there is considerable immunity. These observations confirm our previous *in vivo* findings in experimental animals (Taylor, Ahuja and Singh, 1936) that for practical purposes the adoption of a six-day period for quarantine regulations is probably suitable although it appears that a high degree of immunity would be obtained in 8 to 10 days. The immunity conferred by vaccination appears to be short-lived but without knowing the precise relationship of bactericidal titre and resistance to experimental infection it is not possible to lay down a definite period.

THE EFFECT OF RE-VACCINATION SIX MONTHS AFTER THE PRIMARY DOSE.

Nine subjects who had previously been vaccinated with a single dose of 1 c.c. of cholera vaccine were given a second dose of 1 c.c. six months later and their sera tested for bactericidal response ten days after this secondary stimulus. The results are shown in Table IV:—

TABLE IV.

Bactericidal response in volunteer number:—	19	20	21	4	2	8	1	6	3
Pre-vaccination titre.	45	Nil	15	15	15	45	135	15	45
Titre 10 days after primary dose of 1 c.c.	11,000	33,000	1,200	1,200	33,000	11,000	33,000	11,000	33,000
Titre six months after primary dose.	400	135	15	45	400	400	135	400	400
Titre 10 days after secondary dose.	3,600	1,200	400	135	1,200	1,200	400	400	400

The results show that the primary response after a single dose of 1 c.c. of cholera vaccine is brisk and results in high vibriocidal titre 10 days after vaccination, while the secondary response following re-vaccination six months later is poor and does not increase the vibriocidal power of the serum to any very marked extent compared to the effect produced by the primary stimulus.

CORRELATION BETWEEN BACTERICIDAL RESPONSE AND RESISTANCE TO INFECTION.

It may be argued that although a definite increase occurs in bactericidal titre of a serum after cholera vaccination it does not necessarily follow that the serum

is capable of affording protection. To settle this point the following *in vivo* experiments were carried out.

Four samples of sera of bactericidal titres 1 : 15, 1 : 400, 1 : 3,600 and 1 : 100,000 were tested for prophylactic protective value according to the method described below :—

Guinea-pigs of 350 g. to 450 g. body-weight were injected intravenously with 1.0 c.c., 0.5 c.c., 0.1 c.c., 0.01 c.c., and 0.001 c.c. of the types of sera under test, the total volume injected being brought up to 1.0 c.c. Four hours later all the animals were injected intraperitoneally with a challenge dose of a mucinized suspension of *V. cholerae*. This was prepared from six-hour-old agar cultures washed in normal saline. The concentrated vibrio suspension was standardized to 20,000 million organisms per c.c. (Brown's opacity-standards) using nutrient broth as the diluent. To this vibrio suspension was added an equal quantity of 5 per cent mucin prepared according to the method described by Miller (1934-35). A constant dose of 1 c.c. of this mucinized suspension was used as the challenge dose.

Table V shows the results of protective tests :—

TABLE V.

Relationship of bactericidal power of serum and its passive protective value in guinea-pigs.

Dose of serum injected intravenously, c.c.	SERA OF IMMUNIZED SUBJECTS.						NON-IMMUNE SERUM CONTROL.	
	SERUM I. TITRE 1 : 100,000.		SERUM II. TITRE 1 : 3,600.		SERUM III. TITRE 1 : 400.		SERUM IV. TITRE 1 : 15.	
	Number of guinea-pigs.	Deaths in 72 hours.	Number of guinea-pigs.	Deaths in 72 hours.	Number of guinea-pigs.	Deaths in 72 hours.	Number of guinea-pigs.	Deaths in 72 hours.
1	3	0	5	0	3	2	5	5
0.5	3	0	9	0	4	3	5	5
0.1	7	0	9	1	4	4	—	—
0.01	4	0	7	5	4	4	—	—
0.001	4	3	4	4	—	—	—	—
0.0001	3	3	—	—	—	—	—	—

The significance of the correlation between vibriocidal titre and the passive protective value of the serum of a vaccinated subject is obvious from the findings recorded in the above experiment. The results show that 0.01 c.c. of the serum of a vaccinated subject (bactericidal titre 1 : 100,000) is capable of affording

protection to guinea-pigs against intraperitoneal test infection with homologous live vibrio cultures, a property not possessed by 100 times that dose of a serum from a non-vaccinated subject. It is also obvious that the higher the vibriocidal titre of the serum the greater is its prophylactic protective value.

THE 'PROTECTIVE' RESPONSE EVOKED BY VACCINES PREPARED FROM
SINGLE SUBTYPE STRAINS AND MIXED INABA AND OGAWA
STRAINS OF *V. cholerae*.

A number of sera of immunized human subjects were tested for their protective value ten days after cholera vaccination. The results were more or less similar to those shown in Table VI. Only a representative table is presented.

TABLE VI.

Prophylactic protective power of sera of vaccinated subjects.

Source and particulars, etc.	CHALLENGE DOSE INABA.				CHALLENGE DOSE OGAWA.			
	Dose in c.c.	Number of guinea-pigs injected.	Number surviving.	50 per cent. end-point dose.	Dose in c.c.	Number of guinea-pigs injected.	Number surviving.	50 per cent. end-point dose.
Serum, human subject, vaccinated with Inaba vaccine; vibriocidal titre: 1/33,000 Inaba, 1/11,000 Ogawa; agglutinin titre: 25/50tr. Inaba, 1/225tr. Ogawa.	0.20	5	4	0.08	0.20	5	3	0.088
	0.10	5	3		0.10	5	3	
	0.05	5	2		0.05	5	2	
	0.01	5	0		0.01	5	0	
Serum, human subject, vaccinated with Ogawa vaccine; vibriocidal titre: 1.11,000 Inaba, 1/11,000 Ogawa; agglutinin titre: 1/25tr. Inaba, 1/125tr. Ogawa.	0.20	5	2	0.2	0.20	5	4	0.084
	0.10	5	0		0.10	5	4	
	0.05	5	0		0.05	5	0	
Serum, human subject, vaccinated with mixed Inaba and Ogawa vaccine; vibriocidal titre: 1/11,000 Inaba, 1/11,000 Ogawa; agglutinin titre: 25/50 Inaba, 50/125 Ogawa.	0.20	5	5	0.026	0.20	5	5	0.033
	0.10	5	4		0.10	5	4	
	0.05	5	5		0.05	5	4	
	0.01	5	0		0.01	5	0	
Serum, human subject, not vaccinated; vibriocidal titre: 1/15 Inaba, 1/15 Ogawa; agglutinin titre: 1/25 Nil Inaba, 1/25 Nil Ogawa.	1.0	5	0	...	1.0	5	0	1 c.c.
	0.5	5	0	...	0.5	5	0	

These results confirm the findings of the previous experiment that following cholera vaccination there develop in the serum of vaccinated human subjects protective substances capable of affording passive immunity to guinea-pigs against lethal infection with live virulent cultures of *Vibrio cholerae* and show that the serum of a subject vaccinated with a mixed Inaba and Ogawa vaccine possesses passive protective substances in greater concentration than the serum of a subject vaccinated with a single subtype vaccine, either Ogawa or Inaba.

ACTIVE IMMUNIZATION OF GUINEA-PIGS WITH INABA, OGAWA AND INABA-OGAWA VACCINES.

Active immunization of experimental animals, usually mice or guinea-pigs, with cholera vaccine has in the past been considered as affording a good index for estimating the antigenic value of strains used in the preparation of cholera vaccines. A number of experiments have been carried out on these lines during the last two years. The results of one such experiment are given below:—

Guinea-pigs of 300 g. to 400 g. body-weight were vaccinated subcutaneously with two doses, 0.5 c.c. and 1 c.c., of cholera vaccines prepared from Inaba and Ogawa subtypes of *V. cholerae*. Ten days after the second immunizing dose the animals were given a challenge dose intraperitoneally of mucinized suspensions of live virulent smooth strains of *Vibrio cholerae* and mortality recorded during the next 96 hours.

Number of guinea-pigs immunized	...	90
Number of guinea-pig controls	...	30

Vaccine used.	Number of guinea-pigs immunized.	Infecting strain.	Survivals up to 96 hours.	Percentage of survival.
Inaba 56 : B ...	15	Ogawa 2856	14	93
Inaba 56 : B ...	15	Inaba Ki	14	93
Ogawa 16 : A ...	14	Ogawa 2856	14	100
Ogawa 16 : A ...	15	Inaba Ki	13	89
Inaba + Ogawa ...	15	Ogawa 2856	15	100
Inaba + Ogawa ...	15	Inaba Ki	15	100
Controls ...	15	Ogawa 2856	Nil	Nil
Controls ...	15	Inaba Ki	Nil	Nil

These results do not show if any differences exist in the antigenic value of different subtypes of *V. cholerae*. An Inaba strain vaccine affords just as good protection against Inaba infection as against Ogawa infection and vice versa. The mixed vaccine is slightly better but there is no significant difference in the immunizing power of either Inaba or Ogawa or a mixed Inaba and Ogawa vaccines.

DISCUSSION.

Active immunization of mice or guinea-pigs is employed in a number of laboratories as the sole criterion for finding the superiority of one vaccine over another or the relative merits of strains used in the preparation of cholera vaccine. In our hands the results of this method of assay have not shown the differences in the antigenic value of strains which are so clearly demonstrated by using the serum of vaccinated subjects in passive protection tests in guinea-pigs and it is considered that the latter method is of greater value as an index of assessment than active immunization of experimental animals.

It is admitted, however, that none of these tests are sufficiently sensitive to represent immunity response after cholera vaccination, the reason being that *V. cholera* is not a natural infector of experimental animals. In testing the relative merits of strains or different types of cholera vaccine all available methods should be employed. As criteria we consider agglutination tests as the least sensitive. Bactericidal response runs approximately-parallel to passive protective power and this is a more trustworthy measure of antibody production than the estimation of circulating agglutinins, the level of which is generally so low in experimental animals and particularly in man that it is impossible to assess the degree of immunity by this *in vitro* test. Passive protection test is the most sensitive method available in the present state of our knowledge, for demonstrating difference in the immunizing value of vibrio strains.

SUMMARY AND CONCLUSIONS.

1. These observations show that following cholera vaccination the serum of a vaccinated human subject acquires a specific and well-marked vibriocidal property which is lacking prior to vaccination.

2. The vibriocidal response evoked by cholera vaccine although specific for the race *V. cholera* is not specific for the particular subtype of *V. cholera* used in the preparation of the vaccine. The titre for the homologous subtype is, however, generally higher than for the heterologous subtype.

3. Vibriocidal substances appear on the 3rd day and attain their maximum concentration on the 8th day after vaccination. By the end of the 30th day there is a definite fall in vibriocidal titre. As the period increases there is a progressive loss in titre which by the 100th day is only a little higher than the pre-vaccination level.

4. Re-vaccination after six months does not increase the vibriocidal power of the serum to any marked extent.

5. Agglutinin development after cholera vaccination is very feeble, the titre hardly rising from the pre-vaccination level of 1/25 or less to a maximum of 125/250 ten days after vaccination. The level of circulating agglutinins is generally so low that it is not possible to assess the degree of immunity by this method.

6. The infection-resisting capacity of protective substances present in the sera of vaccinated subjects is higher for homologous than for heterologous subtype infection.

7. The prophylactic protective response evoked by a mixed Inaba *plus* Ogawa vaccine is higher than the response evoked by single subtype vaccines. On the basis of these findings the use of both subtypes of *V. cholerae* for the preparation of prophylactic cholera vaccine would be more satisfactory than the use of either an Inaba or an Ogawa subtype alone.

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THE INCIDENCE OF MURINE TYPHUS AMONGST WILD RODENTS IN POONA AND BOMBAY.

BY

M. G. P. STOKER, M.D. (Cambridge),
(late CAPTAIN, R.A.M.C.).

[From the General Headquarters (India) Base Typhus Research Team. Poona.]

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INTRODUCTION.

DURING the recent war, sporadic cases of murine typhus occurred amongst military personnel stationed at Poona. It was, therefore, decided to investigate the incidence of this infection amongst local rodents and their ectoparasites.

Covell (1936) carried out an investigation of this nature in the Simla Hills, where he succeeded in isolating cross-immunizing strains of rickettsiae from rats and rat-fleas and performed Weil-Felix tests on rat sera. Other serological surveys of larger numbers of rodents, using Weil-Felix tests, have been performed by Smith and Mehta (1937) also in the Simla Hills, and by Shortt and d'Silva (1936) at Kasauli, and Shortt (1936) in Madras. Recently, however, Brigham and Bengston (1945) have shown that complement-fixation tests with murine rickettsial antigen on wild rat sera give a much higher correlation with strain isolation than the Weil-Felix reaction, and Pollard and Augustson (1945) have described a method of surveying rodents by this technique.

At Poona, therefore, the complement-fixation test was used to determine the incidence of murine rickettsial infection amongst locally trapped rodents, and attempts were made to isolate and identify strains of rickettsiae from these rodents and their ectoparasites. At the same time a number of Bombay rat sera were also examined by complement-fixation tests in order to compare the incidence of infection in the two localities.

MATERIAL AND METHODS.

An arrangement was made, whereby numbers of live rodents, trapped in Poona Cantonment, were delivered to the laboratory every day for a four-month period

commencing 11th May, 1946. In addition to rats, bandicoots and voles were also included in the specimens; these were kept separate where possible, but otherwise dealt with in the same way as the rats.

As soon as the rats were brought to the laboratory the ectoparasites were removed in the following way: the cage was placed in a sink containing enough water to cover the bottom and the animals were anaesthetized in the cage by covering it with an ether-soaked cloth. The ectoparasites, which immediately left the anaesthetized animals, fell into the water in the sink from which they were very easily recovered. The animals were then removed from the cage and examined for further parasites. Most of the parasites were kept for attempts to isolate strains of rickettsiae and for this they were separated into groups of fleas and mites. They were stored at 20°C. and batches of 38 or more fleas or mites were used to make suspensions. The parasites were ground in saline and centrifuged at 100 r.p.m. for 2 minutes; 5 c.c. of the supernatant fluid were inoculated intraperitoneally into guinea-pigs. The parasites collected in the first month of the survey and a few of the latter batches were not used for suspensions but were kept intact in spirit or mounted on a slide, and sent to Dr. Sharif of the Haffkine Institute, Bombay, for accurate identification.

The rodents were identified as far as possible by measurement; they were then bled from the jugular vein and killed. From some the brains were removed with sterile precautions, and batches of 3 to 11 of these were ground with glass, and suspended in saline. After light centrifugation as above, 5 c.c. were inoculated intraperitoneally into a guinea-pig. The specimens of serum obtained from the animals were examined at a dilution of 1:10 by complement-fixation tests with murine rickettsial antigen and also with Rocky Mountain spotted fever antigen in some cases. The technique used was the same as that described in a previous communication (Seaton and Stoker, 1946). On all sera which showed fixation at 1:10 the test was repeated in higher dilutions in order to determine the end-point. Specimens of serum from Bombay rats, trapped and sent to the Haffkine Institute for the detection of plague, were forwarded to the laboratory by Dr. Menezes, and were also examined by the same technique.

RESULTS.

1. *Species of rodents*.—During the four-month period 11th May to 11th September, 1946, 407 rodents were examined. These included 350 rats, mostly *Rattus rattus*, 45 bandicoots, *Bandicoota malabarica* and 12 voles, *Suncus caeruleus*. No *Rattus norvegicus* was captured and two rats which were identified accurately by Dr. Menezes were both *R. rattus*. It is probable, however, that the total included a number of the rat *Gonomys gonomys*. Although this is a field rat, it also enters buildings and it may not have been distinguished owing to its superficial resemblance to *R. rattus*. Females comprised 44 per cent of both the rats and the bandicoots, of which 16 per cent and 33 per cent respectively were pregnant. Of the voles 33 per cent were female but none were pregnant.

Four attempts were made to isolate strains of rickettsiae by intraperitoneal inoculation of guinea-pigs with pooled brain suspensions from these rodents (Table I). With the exception of one batch, the rodents used were taken at random,

TABLE I.

Intraperitoneal inoculation of guinea-pigs with pooled suspensions of rodent brains.

Batch number.	Date of guinea-pig inoculation.	Serial number of rodents.	Results of complement-fixation tests (murine antigen).	Results of inoculation.
1	12th June, 1946	56 (rat)	1 : 10	Developed fever on 14th day and serotal reaction on 15th day. Strain 191 isolated.
		59 "	Nil	
		60 "	Nil	
		61 "	Nil	
		62 "	...	
		63 "	...	
2	7th June, 1946	64 "	Nil	Developed fever on 9th day and serotal reaction on 10th day. Strain 192 isolated.
		55 (bandicoot)	Nil	
		57 "	Nil	
3	15th June, 1946	58 "	Nil	No reaction.
		65 (rat)	Nil	
		66 "	Nil	
		67 "	Nil	
		68 "	Nil	
		69 "	Nil	
		70 "	Nil	
		71 "	Nil	
		72 "	Nil	
		73 "	Nil	
4	21st June, 1946	74 "	Nil	Died with mal-ture on 21st June.
		75 "	Nil	
		89 (rat)	1 : 10	
		91 "	1 : 10	
		94 "	1 : 10 (2+ only)	
		95 "	1 : 10	No reaction.

irrespective of the results of the complement-fixation tests. Batch No. 4, however, was deliberately made up of rats whose sera had shown complement-fixation with murine typhus antigen.

Two strains were isolated: one from batch No. 1 (7 rats) and the other from batch No. 2 (3 bandicoots). The strains were named Poona 191 and 192, respectively, and are described in detail below. It should be noted that serum from one of the 7 rats showed a positive complement-fixation test with murine typhus antigen, but the serological reactions of all three bandicoots were negative. This finding is discussed in the account of the strain.

2. *Ectoparasites*.—All the ectoparasites collected during the earlier part of the survey from 11th May to 3rd June were identified accurately by Dr. Sharif: the 74 specimens in this group were obtained from 51 rats. (There were no bandicoots or voles in this series.)

The parasites were identified as follows:—

<i>Xenopsylla cheopis</i>	♂	8
" "	♀	9
<i>Xenopsylla astia</i>	♂	1
" "	♀	4
<i>Xenopsylla braziliensis</i>	♀	3
<i>Dermonyssus muris</i>		49
TOTAL				74

From 4th June until 22nd August, when ectoparasite collection was stopped, most of the specimens were kept for strain isolation but a proportion were sent for identification. From a total of 278 rats, bandicoots and voles examined during this period, 474 fleas and 210 mites were collected. Fifty of these which were identified included specimens of *Liponyssus nuttali* as well as *D. muris* and *X. cheopis* and one *Polyplax spinulosa*. An unidentified adult tick was also collected but no larval ticks or mites were found. The number of parasites recovered from individual animals varied considerably; one bandicoot yielded 36 fleas, but on another occasion, no parasites at all were found on 12 successive animals (9 rats, 2 voles and 1 bandicoot). Mixing of the ectoparasites occurred if two or more species of rodent were captured and brought to the laboratory in the same cage, but when this cross-infestation could be excluded, it was apparent that both fleas and mites could generally be found on all three hosts.

In attempts to isolate strains of rickettsiæ from these parasites, 5 pools of fleas and one pool of mites were used for preparation of saline suspensions which were inoculated intraperitoneally into guinea-pigs. Details of these experiments are given in Table II.

A strain of rickettsiæ was isolated from batch No. 5 which consisted of 142 fleas. These had been collected from 104 rats, 10 of which had shown a positive

TABLE II.

Intraperitoneal inoculation of guinea-pigs with pooled suspensions of ectoparasites.

Batch number.	Date of guinea-pig inoculation.	Parasites.	Hosts.	Results of inoculation.
1	28th June, 1946	56 fleas	Rats	No reaction.
2	7th July, 1946	38 fleas	Bandicoots	No reaction.
3	11th July, 1946	91 mites	Rats and bandicoots	Developed fever but no scrotal reaction. Further passage produced no reaction.
4	12th July, 1946	95 fleas	Rats	No reaction.
5	14th August, 1946	142 fleas	Rats	Developed fever on 8th day and scrotal reaction on 9th. <i>Strain 193 isolated.</i>
6	23rd August, 1946	38 fleas	Rats and bandicoots	No reaction.

complement-fixation test with murine antigen. The strain 193, which is immunologically related to strain 192 from bandicoots, is described in detail below.

3. *Serological findings.*—Twelve rats and 3 bandicoots were excluded because their sera were anti-complementary. The results of complement-fixation tests with murine typhus antigen on sera from the remaining 392 rodents were as follows:—

Rat sera	...	338 examined	32 positive at 1 : 10 or higher (9.5%)
Bandicoot sera	...	42 "	3 " " " " (7.1%)
Vole sera	...	12 "	All negative.

TOTAL	...	392	35 positive.
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Table III shows the titres obtained:—

TABLE III.

Results of complement-fixation tests with murine typhus antigen on Poona rodent sera.

Rodent.	Number examined.	Number positive according to titre :				Total positive.	Percentage positive.
		1 : 10	1 : 20	1 : 40	1 : 80		
Rats	...	20	6	3	3	32	9.5
Bandicoots	2	...	1	3	7.1
Voies	Nil	...
TOTAL	...	20	8	3	4	35	8.9

These were generally low; less than half (43 per cent) were over 1 : 10 and none higher than 1 : 80. There was no significant difference in the sex distribution of the positive sera, nor was there any relationship with the presence or absence of pregnancy.

Weil-Felix and rickettsial agglutination tests with murine typhus suspensions were also performed on 9 positive rat sera and one positive bandicoot serum. All agglutination tests were negative except in the case of one rat serum which had fixed complement to a dilution of 1 : 80, and which also agglutinated *Proteus* OX19 suspension to 1 : 800 (zone of inhibition to 1 : 100). The same serum failed to agglutinate murine rickettsial suspension.

In an attempt to identify the reservoir of Indian 'tick typhus', 74 rat and 17 bandicoot sera were also tested for complement-fixing properties in the presence of Rocky Mountain spotted fever antigen, but these tests were all negative.

4. *Comparative survey of Bombay rats.*—For comparison, specimens of serum from 77 rats captured in Bombay between 19th April and 29th July, 1947, were also examined. These rats were all identified but ectoparasites were not collected.

After excluding two anti-complementary sera, the results of complement-fixation tests with murine typhus antigen on the remaining 75 are shown in Table IV. The total proportion of positives (9.3 per cent) approximated closely to the total for Poona rats (9.5 per cent). It appeared that larger numbers of *R. rattus* had been infected than the other species but the figures are scarcely significant.

TABLE IV.

Results of complement-fixation tests with murine typhus antigen on Bombay rat sera.

Species.	Number examined.	Number positive according to titre :		Total positive.	Percentage positive.
		1 : 10	1 : 20		
<i>R. rattus</i> ...	19	3	2	5	26
<i>R. norvegicus</i> ...	9	Nil	...
<i>G. gonomys</i> ...	47	1	1	2	4
TOTAL ...	75	4	3	7	9.3

Dr. Soman of the Haffkine Institute (personal communication, 1946) succeeded in isolating a strain of rickettsiae from the brains of 4 *R. rattus* sera, from three of which fixed complement with murine typhus antigen. The pooled brains were inoculated into a guinea-pig which developed fever six days later. On the 9th day it was killed and passage made to two white rats. These showed no reaction, but when material from one rat was passaged back into a guinea-pig, the latter developed

fever and scrotal swelling on the 11th day and rickettsiae were demonstrated in peritoneal smears when the animal was killed.

5. *Strains isolated in Poona: Strain 191.*—This strain was isolated from 7 rats (*R. rattus*) captured between 4th June, 1946 and 12th June, 1946. Serum from one of them showed a positive complement-fixation test with murine typhus antigen at 1:10, while the remaining 6 were negative.

A pooled suspension of the brains of these rats was inoculated intraperitoneally into a guinea-pig on 12th June, 1946. On 26th June, 1946, after an incubation period of 14 days, the guinea-pig developed a temperature of 103.5°F. and on the following day there was well-marked scrotal reaction. The animal remained febrile until it was killed on the 16th day after inoculation. On post-mortem examination there was a peritoneal exudate and the tunica vaginalis was hyperæmic and œdematous. Giemsa-stained smears from the latter showed intra-cytoplasmic inclusion indistinguishable from rickettsiae. The brain, spleen and testes were used for inoculation of two more guinea-pigs. Neither of these showed any scrotal reaction: one developed a temperature of 102.7°F. on the 12th day and this animal was allowed to recover, but examination of the serum 55 days later showed no complement-fixing antibodies against murine typhus. The second guinea-pig became slightly febrile with a temperature of 103°F. on the 12th day. When this animal was killed on the 14th day, there was only a slight peritoneal exudate, but smears from the tunica vaginalis, which appeared macroscopically normal, showed scanty rickettsiae.

In these and subsequent passages, the reaction in guinea-pigs was very variable. There was only a typical infection with scrotal reaction in passages 1 and 6, but rickettsiae were seen in smears from passages 1, 2, 4 and 6. Complement-fixation tests with murine typhus antigen were performed on serum from passages 1, 2, 6 and 7, and these were all negative. Serum from passage 7 was also negative with Rocky Mountain spotted fever antigen. These negative results might be due to the fact that specimens were taken only 2 or 3 days after the onset of infection.

The only cross-immunity test performed was inconclusive, due to an absence of a standard response in the control animal. The test animal had been inoculated with the Wilmington strain of murine typhus on 1st July, 1946, and re-inoculation with strain 191 on 23rd August, 1946, caused no reaction at all, but the febrile response in the normal control animal was also very slight and there was no scrotal reaction. No obvious change was found on post-mortem examination in either animal and smears from both were negative.

The only other animals inoculated were two mice, neither of which showed any infection.

Although no conclusive cross-immunity test has been performed it seems probable that the strain is one of murine typhus and this is borne out by the positive-fixation test on serum from one of the original rats.

Strain 192.—This strain was isolated from 3 male bandicoots (*B. malabarica*) captured on 4th, 6th and 7th June, 1946, whose serum showed no complement-fixation with murine typhus antigen.

A pooled suspension of the brains of the animals was inoculated intraperitoneally into a guinea-pig on 7th June, 1946. The guinea-pig developed a temperature of 103.5°F. on the 9th day after inoculation and a scrotal reaction was apparent on the following day. When killed on 20th June, 1946, 13 days after inoculation, a peritoneal exudate was present, but the tunica vaginalis was only slightly inflamed. Peritoneal smears showed large numbers of rickettsiae and the serum at 1:10 fixed complement in the presence of murine typhus antigen. A suspension of brain, spleen and testes was used for passage to two more guinea-pigs, both of which developed fever and a scrotal reaction on the 6th day after inoculation. One animal was allowed to recover and was bled on the 21st day after inoculation. Complement-fixation and rickettsial agglutination tests with murine rickettsiae were both positive at 1:160 and 1:600 respectively. There was also cross-agglutination with epidemic typhus to 1:100, but no cross-fixation with Rocky Mountain spotted fever antigen. The second guinea-pig was killed on the 9th day after inoculation, and brain, spleen and testes were used for further passages. In subsequent passages, nearly all the guinea-pigs developed a well-marked scrotal reaction and it was easy to demonstrate rickettsiae in smears from peritoneum or tunica vaginalis. Two white mice were also inoculated from the 2nd passage but they remained well, and when killed 17 days later, showed no sign of infection.

Material from the 7th passage was used for inoculation of an animal previously inoculated with the Wilmington strain on 24th May, 1946, but both the test and control animals developed a severe reaction on the 3rd day from which the control animal died, presumably due to contamination of the inoculum, although the responsible organism could not be isolated.

The immune guinea-pig recovered and when it was killed on the 12th day after inoculation rickettsiae were present in smears, but in the absence of a satisfactory control, no conclusions could be drawn. The test has not yet been repeated owing to loss of the Wilmington strain.

A guinea-pig which had recovered from the 5th passage was subsequently shown to be immune to strain 193 isolated from rat-fleas described below:—

In view of the serological reactions in guinea-pigs it is probable that this strain is also one of the murine typhus. The negative complement-fixation test in the original bandicoots is difficult to explain, although it is possible that the animal concerned had only become infected a few days before it was killed and sufficient time had not elapsed for the development of complement-fixing antibodies. Pollard and Augustson (*loc. cit.*) showed that when white rats are infected with murine typhus, the complement-fixation test does not become positive for 14 days. It is interesting to note that Brigham and Bengston (*loc. cit.*) also isolated a strain of murine typhus from a wild rat whose serum showed a negative complement-fixation test.

Strain 193.—This strain was isolated from a batch of 142 fleas collected from Poona rats between 19th July, 1946 and 13th August, 1946. A suspension of the fleas was inoculated intraperitoneally into a guinea-pig on 14th August, 1946; 8 days later the animal's temperature rose to 103.5°F. and the following day a scrotal reaction was observed. When killed on the 10th day after inoculation there

was hyperæmia of the peritoneum but little exudate. The tunica vaginalis was also inflamed but this was not so obvious in the visceral as in the parietal portion where the changes also involved the subcutaneous tissue of the scrotum. Smears showed scanty rickettsiæ: no complement-fixing antibodies against murine typhus or Rocky Mountain spotted fever antigens were detected in the serum.

A suspension of brain, spleen and testes was passaged to another guinea-pig, which, after an incubation period of 7 days, also developed fever and a scrotal reaction. This animal was killed on the 10th day after inoculation and showed a considerable peritoneal exudate and marked inflammation of the tunica vaginalis. The complement-fixation test was again negative with both antigens.

The 3rd passage was in a normal guinea-pig and also in one which had recovered from strain 192 (passage 5). The immune animal showed no reaction at all, whereas the normal guinea-pig developed a typical fever and scrotal reaction commencing on the 4th day after inoculation. Post-mortem examination 3 days later showed a marked inflammation of the peritoneum and tunica vaginalis and large numbers of rickettsiæ were seen in smears. It appears, therefore, that strains 192 and 193 are immunologically related.

DISCUSSION.

The object of this investigation was to study incidence of murine typhus amongst Poona and Bombay rodents. By complement-fixation tests it was found that 9.5 per cent of 338 rats and 7.1 per cent of 42 bandicoots showed evidence of previous murine infection. Rickettsial infection in these rodents was confirmed by the isolation of strain 191 from rats and strain 192 from bandicoots. Although no conclusive cross-immunity tests have been completed, the source, behaviour in experimental animals and serological results suggest that these were strains of murine typhus.

The incidence amongst Bombay rats was approximately the same as in Poona and an orchitic strain was isolated there as well. This confirms Patel's deduction that murine typhus occurs in Bombay.

The negative Weil-Felix in 9 out of 10 sera which had shown a positive complement-fixation test is not unexpected, and the one serum which agglutinated *Proteus* OX19 may have been from a rat which had been only recently infected. The absence of rickettsial agglutination by all ten sera is more difficult to explain, particularly as van Rooyen, Bowie and Crikorian (1944) used this test successfully for the examination of rat sera in the Middle East.

Although the bandicoot has not previously been incriminated as a reservoir of murine typhus, the observation is not unexpected in view of the large number of rodents that have already been shown to be naturally infected. Brigham and Dyer who found 8 species of American rodents capable of transmitting murine typhus suggest that all rodents are susceptible.

It should be noted that the bandicoot *Isodon torosus* from which Burnet and Freeman (1939) isolated strains of Q fever in Australia is in no way related to *B. malabarica*. Blake, Maxey, Sadusk, Kohls and Bell (1945) succeeded in isolating strains of scrub typhus from trombiculid mites infesting the bandicoot *Echymipera*

cocherelli, but there was no evidence that the bandicoot itself was infected. None of the voles examined in Poona showed evidence of murine typhus, but the number was too small to exclude the possibility of voles being sometimes infected.

A further strain, 193, was isolated from fleas on the Poona rodents and strain 192, from bandicoots, immunized a guinea-pig completely against this strain. No strain was isolated from the mites which also parasitized the animals, but it is possible that they also act as vectors: *Liponyssus bacoti* Hirst has already been shown capable of transmitting murine typhus by Dove and Shellmire (1931).

Although about 9 per cent of rats and bandicoots showed positive complement-fixation tests it does not follow that this proportion were infective at the time. The test probably remains positive for the rest of the animals' life, and the low titres, which are similar to those obtained by Woodward, Phillip and Loranger (1946) in the Philippines, suggest that most of the infections were not recent. Nevertheless, the blood of experimentally infected rats has been shown to be infective for at least a month and even as long as a year after inoculation, and the relative ease with which strains were isolated from the Poona rodents suggests that at any one time the proportion of infected animals is not negligible.

Contact with these rodents and their ectoparasites accounts for the incidence of murine typhus amongst troops in Poona. The disease is mild and there are probably many undiagnosed cases which are not admitted to hospital. Prevention of the disease will be best effected by elimination of the rodent population: in areas where anti-plague measures are most effective the incidence of murine typhus should also be low.

SUMMARY.

1. The incidence of murine typhus amongst wild rodents in Poona and Bombay was assessed by complement-fixation tests with murine rickettsial antigen.

2. Evidence of previous infection was found in 9.5 per cent of 338 rats and 7.1 per cent of 42 bandicoots from Poona, and in 9.3 per cent of 75 rats from Bombay.

3. Rickettsial infection amongst these rodents in Poona was further confirmed by the isolation of two strains from the pooled brains of rats and bandicoots respectively, and further strain from rat-fleas. Although no conclusive cross-immunity tests were completed, it seems probable that they were all strains of murine rickettsiae and this conclusion is supported by the fact that previous infection with the bandicoot strain completely protected a guinea-pig against the flea strain.

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THE NUTRITIVE VALUE OF HYDROGENATED VEGETABLE OILS.

THE DIGESTIBILITY OF GROUND-NUT (*ARACHIS HYPOGEA*) OIL HYDROGENATED TO DIFFERENT DEGREES OF HARDNESS.

BY

U. C. MISRA,

AND

V. N. PATWARDHAN.

(From the Nutrition Research Laboratories, Indian Research Fund
Association, Coonoor, South India.)

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THE question of the nutritive value of hydrogenated vegetable oils has recently assumed importance in India owing to the great expansion of the hydrogenation industry and increased consumption of the product as food. The unfortunate choice of the name of Vegetable Ghee (the use of which is no longer permitted) for the product has created a wrong impression in the minds of the lay public and a certain amount of controversy in the lay press in which the scientific workers could not take part has helped to arouse the public interest. It was felt necessary, therefore, to investigate carefully some aspects of the nutritive value of hydrogenated oils.

The digestibility of a foodstuff is one of the factors which determine its nutritive value. The refined vegetable oils have been found to be digestible to the extent of 95 to 100 per cent (Basu and Nath, 1946; Patwardhan and Nhavi, 1946—unpublished results). The rise in the melting point due to an increase in saturation and the formation of iso-oleic acids are the two changes taking place during hydrogenation which might affect the digestibility of the hydrogenated fat.

Several studies on digestibility of fats carried out on rats, guinea-pigs and human beings have been reported. Langworthy and Holmes (1915, quoted from

Holmes and Deuel, 1920) have observed that in human beings the digestibility coefficient of the fats melting over 37°C. varied inversely with their melting point. Deuel and Holmes (1921) found that some hydrogenated fats melting from 52°C. to 60°C. gave low values for digestibility. On the other hand Hoagland and Snider (1943a) working with rats found no relation between the melting point of a fat and its digestibility between 26°C. and 47°C. In a later communication these authors (Hoagland and Snider, 1943b) reported a digestibility of 100 per cent for Trilaurin (m.p. 48°C.) and Trimyristin (m.p. 56°C.), 84 per cent for Tripalmitin (m.p. 65°C.) and 6 to 8 per cent for Tristearin (m.p. 70°C.)

McCay and Paul (1938) have observed a specific difference in the digestibility of fats. According to them, the guinea-pig cannot utilize the high melting fat to the same extent as does the rat. It is quite probable that a similar difference may exist between man and rat, although no evidence is available. Basu and Nath (*loc. cit.*) reported in human beings a 90 per cent digestibility of a sample of hydrogenated ground-nut oil as compared to the values of 94 to 99 per cent observed with other vegetable oils. They ascribed this small difference to the high melting point of the former.

The authors of the present paper felt that if comparative digestibility trials were made on one fat hydrogenated to different degrees of hardness it should be possible to get a more correct idea of the influence of temperature on digestibility than by working with different fats of varied composition and different melting point.

It was possible to obtain through the generosity of Messrs. Hindustan Vanaspati Manufacturing Co., Ltd., Bombay, genuine samples of ground-nut oil thus hydrogenated. The samples had melting points of 29.5°C., 37°C., 40°C., 41.5°C., 43.5°C., and 49°C., as confirmed in this Laboratory.

Another aspect which also deserved attention at the same time and to which a reference has already been made was that of the influence of glycerides of iso-oleic acids on the digestibility of hydrogenated fats. It is well known that during hydrogenation unnatural isomers of oleic acid are formed (Hilditch, 1941). These are unsaturated acids, but they are solid at room temperature and their sodium salts do not form a good lather. There appears to be a point of view that owing to these latter properties with their adverse influence on emulsification, the glycerides of iso-oleic acids should be less digestible than those of oleic acids. Since the extent of the formation of the iso-oleic acids depends upon the conditions of hydrogenation (Hilditch, 1938; Langton, 1932) the products made under different conditions should vary in their iso-oleic acid content and hence in their digestibility. The Hindustan Vanaspati Manufacturing Co., Ltd., again kindly placed at the disposal of the authors two samples of hydrogenated ground-nut oil melting at 39°C. and 42°C. and containing different amounts of iso-oleic acids. The iso-oleic acid content of one was 9 per cent and of the other 18 per cent. The digestibility of these fats was tested together with that of refined ground-nut oil with a different set of animals.

EXPERIMENTAL.

Six albino rats of weights varying from 140 g. to 160 g. were kept in metabolism cages and fed first on a fat-poor diet consisting of a mixture of 49 per cent of rice

TABLE.

Comparative digestibility of partially hydrogenated ground-nut oil.

Particulars.	Melting point of fat, °C.	Number of animals.	Food consumed.	Fat in food.	Faeces, dry weight.	Fat in faeces.	Fat retained.	Digestibility percentage.
Fat-poor diet	...	6	40.8	0.306	2.095	0.088
Partially hydrogenated ground-nut oil.	29.5	6	35.28	8.045	2.022	0.368	7.677	95.45
	37	6	40.95	9.337	3.356	0.581	8.750	93.75
	40	6	42.00	9.576	3.644	0.505	9.071	93.81
	41.5	6	40.25	9.169	3.378	0.405	8.674	94.79
	43.5	6	45.05	10.269	4.280	0.636	9.633	93.72
Ground-nut oil	49	6	40.33	10.564	4.691	0.776	9.788	92.45
	...	5	33.74	7.759	2.436	0.313	7.446	95.96
Partially hydrogenated ground-nut oil with iso-oleic acids content at 9 per cent.	...	6	31.77	7.308	2.615	0.425	6.883	94.16
Partially hydrogenated ground-nut oil with iso-oleic acids content at 18 per cent.	...	6	31.77	7.308	2.610	0.432	6.876	94.18

flour, 25 per cent casein, 20 per cent starch and 6 per cent salt mixture. The first three materials had been extracted twice with chloroform. Even after this treatment the diet was not completely free from the lipids as was found during the determination of ether extractives by exhaustive extraction of a small sample. This diet was fed for 8 days, the faeces being collected for the last four days. The faeces were dried and analysed for fat by exhaustive extraction with ether.

The same rats were then fed the experimental diet which was prepared by substituting the starch in the diet by fat at 22.8 per cent level. The experiment was so designed that each of the six rats received a different fat. When the first period was over the fat in the diet of each rat was changed. This arrangement was followed till all six rats had received every one of the fats in turn.

DISCUSSION.

The results given in the first line in the Table are obtained on a diet containing 0.654 per cent of ether extractive. It was difficult to make the diet in bulk completely free from fat. It was felt, however, that this low fat content was not likely to vitiate the results. In the calculation of the digestibility the so-called endogenous fat has not been subtracted from the faecal fat observed on fat diets. So the figure obtained is the apparent digestibility. If allowance is made for the former the true digestibility will still be higher, being nearer 100 per cent.

The Table clearly shows that there is no appreciable decrease in digestibility of hydrogenated fats melting between 29.5°C. and 49°C. The small differences observed have been found statistically not significant. The values vary within the limits of experimental error and normal biological variation. Fats with melting points higher than 49°C. were not tested as it was hardly likely that such fats would ever be made or sold for human consumption. While thus it seems desirable to keep the melting point of the hydrogenated fats fairly low, one need not be dogmatic about the maximum being kept below 37°C.

The digestibilities of the hydrogenated ground-nut oil containing different proportions of iso-oleic acids are similar and very near to that of ground-nut oil itself. Thus, the presence of nearly 20 per cent of fatty acids possessing properties unfavourable to emulsification in the gastro-intestinal tract did not appear to have influenced the digestibility.

SUMMARY.

1. The comparative digestibility of ground-nut oil and the same hydrogenated to different degrees of hardness and under conditions so as to yield fats with high and low content of iso-oleic acids has been tested.
2. The digestibility of hydrogenated ground-nut oil of m.p. 29.5°C. did not differ significantly from that of the product melting at 49°C.
3. The hydrogenated products of ground-nut oil containing different proportions of iso-oleic acid had the same digestibility as one another and as that of ground-nut oil itself.

The authors have great pleasure in thanking the Hindustan Vanaspati Manufacturing Co., Ltd., Bombay, for their generosity in placing at their disposal free

of charge various samples of fats mentioned in the text for the purposes of the present investigation.

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THE RELATION OF VITAMIN A TO WHITE CELLS IN HUMAN BLOOD AND NORMAL WHITE CELL COUNTS IN THE PUNJAB.

BY

MANSOOR UL HASSAN,
MOHAMMED IBRAHIM,

AND

LAL CHAND KHANNA.

*(From the Department of Physiology, King Edward Medical College,
Lahore.)*

[Received for publication, August 4, 1947.]

Part I.

THE RELATION OF VITAMIN A TO WHITE CELLS IN HUMAN BLOOD.

INTRODUCTION.

THE exact rôle of vitamin A in the physiology of the body is not known. The lack of this vitamin brings about some characteristic, histological and physiological changes such as the keratinization of the epithelial surfaces and impairment of dark adaptation. Lowered local resistance to infection is the most important effect resulting from vitamin A deficiency. The general defence mechanisms of the body in relation to vitamin A as shown by the hæmogram of blood and susceptibility to bacterial toxins have been studied by a number of workers (Abbot. Ahmann and Overstreet, 1939; Brenner and Roberts, 1943; Blanchard and Harper, 1940; Torrance, 1936, quoted by Bicknell and Prescott, 1946). According to Bicknell and Prescott (*loc. cit.*), the general defence mechanisms are not impaired so that the name anti-infective vitamin is too broad in its implications. The picture, however, is not so clear-cut and such conclusions may be regarded as unwarranted.

The response of the defence mechanisms to the threshold deficiency of the vitamin has not been studied as in the case of Abbot *et al.* (*loc. cit.*). In the present investigation, a study of vitamin A and its relation to white cells in the blood has been made in normal persons, without the clinical manifestations of vitamin A deficiency.

EXPERIMENTAL.

1. *Vitamin A content of blood.*—The method of assay of vitamin A in the blood has been described in a previous communication by the authors (Hassan and Khanna, 1947). The determination of the carotene in the blood was not undertaken in this investigation. After the first extraction with petroleum ether of vitamin A and carotene, the ether was removed and the residue dissolved directly in absolute alcohol. The carotene was eliminated as described previously and vitamin A only was assayed.

The carotene can be better precipitated from the solution in absolute alcohol with a 0.1 per cent solution of sodium chloride. With 0.05 per cent solution, sometimes a colloidal solution is obtained, especially when the temperature is not very low. This can be avoided with 0.1 per cent solution.

2. *Hæmatological technique.*—As a comparative study of the white cells was to be made, it was necessary to reduce to a minimum the inaccuracies due to technique and experimental conditions. Garrey and Bryan (1935) have reviewed in detail the various factors that contribute to the variation of white cells in the blood, and the modifications introduced by different investigators to eliminate these factors. In accordance with the findings of the investigators the following precautions were observed in this investigation:—

(a) The enumeration of white blood cells:

- i. To procure samples representing systematic blood, venous blood was used both for the counting of the white cells and the preparation of blood films.
- ii. A rubber-cap was put on the blood-counting pipette after filling it and continuously rotated for five to seven minutes in a horizontal condition with slight tilting of its ends up and down. This ensured proper mixing.
- iii. Neubauer counting chambers were used. After filling a counting chamber, a considerable quantity of the fluid was expelled from the pipette and the second chamber filled with another drop. The counts of the two chambers were compared with each other. If the difference between the two numbers was more than a few, the process was repeated with a new sample of blood. This ensured a thorough mixing in the pipette and that uniform distribution on the field had been obtained.

(b) The blood films:

The blood films were stained with the combined stains of Giemsa and Leishman to ensure the proper staining of basophils as suggested by Napier and Das Gupta (1945). Two hundred total cells were counted from each of the slides to keep a uniform standard of the differential count.

The time of staining had to be adjusted according to the temperature. The time increased as the temperature decreased.

(c) Experimental conditions :

- i. Fluctuations in the magnitude of the leucocyte count during the different periods of the day is an established fact. According to Boyd (1944) the number of leucocytes varies from hour to hour. It has, therefore, been recommended by Whitby and Britton (1946) to carry out comparative leucocyte counts at the same time of the day. Accordingly, the blood of the subjects was drawn at about 10 a.m. every day.
- ii. Naegeli (1931, quoted by Garrey and Bryan, *loc. cit.*) and many other workers have emphasized the fact that all leucocyte counts should be made in the early morning under condition of rest. The subjects of this study were college students. They were instructed not to indulge in any exercise or extraordinary activity in the morning before coming to the college. The blood was drawn just before the college hours. They were made to sit and rest for ten minutes before giving their blood.
- iii. The blood was always drawn from the subjects in the sitting position after the rest to avoid any variation in the blood count due to posture.
- iv. The subjects were allowed to have a light breakfast early in the morning as they had to attend the college after giving blood. Fasting subjects were preferable but could not be arranged.

3. *The choice of subjects.*—The subjects of this investigation were the students of the K. E. Medical College, Lahore, between the ages of 19 and 22. They were examined physically before drawing their blood. Only normal persons were selected. Cases of local or general infection and those with recent history of illness were excluded. Even mild infections were avoided. The subjects did not show the clinical manifestations of any deficiency disease.

The effect of age, if any, on the vitamin A level or the leucocyte count was eliminated as all the subjects belonged to the same age group.

RESULTS.

The results of the sixty cases examined have been summarized in Table I and graphically shown in the Graph. These values are for the male subjects only.

Vitamin A was assayed by the antimony trichloride method. There is no definite conversion factor to express Blue Units into International Units of vitamin A. Leong (1941) has adopted the conversion factor as 0.6. Heilbron, Jones and Bacharach (1944) have suggested to multiply C.P. values by 30 to convert them into I.U. of vitamin A, which means 1 B.U.=1.2 I.U. The authors have also made similar observations on vitamin A concentrates. A vitamin A concentrate standardized to contain 60,000 I.U. by the manufacturers was found to have

TABLE 1.

	Vitamin A I.U. per 100 c.c. plasma.	Total leucocytes per c.mm. blood.	PERCENTAGE OF DIFFERENT LEUCOCYTES IN NORMAL INDIVIDUALS.				
			Neutro- phils.	Lympho- cytes.	Mono- cytes.	Eosino- phils.	Baso- phils.
Average value ...	125	7,510	55	37	4.5	3.5	0.0.5
Mean deviation ...	± 14	$\pm 1,140$	± 7.0	± 7.0	± 1.5	± 1.5	...
Average range ...	111-139	8,650-6,350	48-62	30-44	3-6	2-5	...

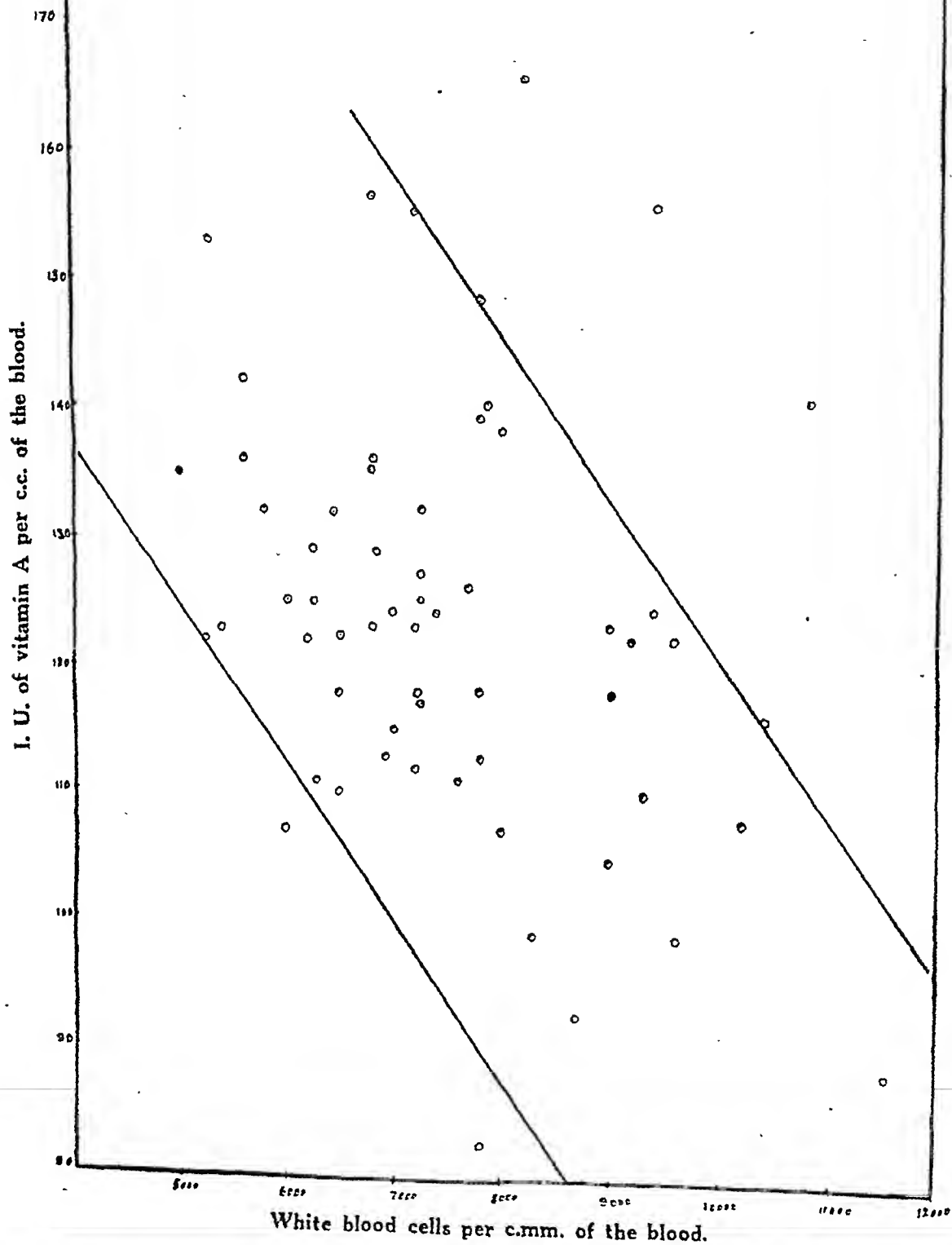
TABLE I—*contd.*

	ARNETH COUNT: PERCENTAGE OF GROUPS.					Weighted mean.
	1	2	3	4	5	
Average value ...	30	39	25	6	0.1	2.1
Mean deviation ...	± 8	± 4	± 5	± 3	...	± 0.2
Average range ...	22-38	35-43	20-30	3-9	...	1.9-2.3

TABLE I—*concl'd.*

	SCHILLING HÆMOGRAM PERCENTAGES.						
	Basophils.	Eosino- phils.	Myelo- cytes.	Juve- niles.	Stab.	Segmented.	Lympho- cytes.
Average value ...	0.0.5	3.5	0.0.5	0.1	15	39	37
Mean deviation	± 1.5	± 4	± 6	± 7
Average range	2-5	11-19	33-45	30-44

VITAMIN A AND WHITE CELLS IN THE BLOOD.



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59,200 B.U. per gramme. In another case, the respective values were 51,360 I.U. and 46,230 B.U. per gramme. The $E_{\frac{1\%}{1\text{ cm.}}}$ value of the latter was found by the authors to be the same as the standardized value. In view of these considerations the authors have taken the conversion factor as 1 to express the Blue Units into International Units.

VITAMIN A LEVEL IN BLOOD.

The average level of vitamin A found in this study is higher than 91 ± 25 , previously found by the authors (Hassan and Khanna, *loc. cit.*). February to May and September to December were the respective periods of the former and the present studies. Thus, vitamin A tends to be low in the spring. This observation, however, needs confirmation with more experimental data, as the subjects of the two studies were not the same. A similar observation was made by Yudkin (1941) in England. In winter vegetables are scarce in England and the low values in the early spring are due to the decreased intake of vegetables. Such an explanation does not hold good here in the Punjab where plenty of vegetables are available all the year round. The analytical data about the dietary habits in the Punjab in different seasons is not available. Nothing definite can be said regarding the choice of food although there is a tendency of greater intake of vegetables, fruit and milk products in summer and of meat, fish, eggs and dry fruit in winter.

VITAMIN A AND BLOOD PICTURE.

1. *Total leucocyte count.*—The blood picture in relation to vitamin A has been summarized in Table II and in the subsequent tables. In Table II the variation of the total number of leucocytes with the rise in vitamin A level has been shown :—

TABLE II.

Vitamin A level I.U. per 100 c.c. plasma.	Number of subjects.	TOTAL LEUCOCYTE COUNT.	
		Average value.	Normal range.
Below normal (110 and below) ...	11	8,630	9,850 to 7,550
Normal—			
i. 111-120 ...	14	7,430	8,250 to 6,600
ii. 121-130 ...	17	7,150	8,200 to 6,100
iii. 131-140 ...	11	7,130	8,250 to 6,000
Above normal (above 140) ...	7	7,350	8,300 to 6,400

It will be seen from Table II that, as the level of vitamin A rises, the leucocyte count tends to lower. The leucocyte count has reached the normal upper limit in cases of subnormal vitamin A level. Within the normal limits of vitamin A level, the corresponding variation of the leucocytes is light but

convincing. With the exception of the small number of cases with vitamin A above normal, the inverse relationship of vitamin A level to the leucocyte count is definite. It is, therefore, to be concluded that vitamin A deficiency in the preliminary stages brings about physiological leucocytosis. It must be distinguished from the leucocytosis caused by infection or a pathological state. Even a mild infection gives the leucocyte count considerably higher and a feature of this type of leucocytosis is the disappearance of eosinophils (Osgood, 1940, quoted by Bicknell and Prescott, *loc. cit.*). In this investigation, the values of both the total leucocyte count and the eosinophils vary within normal limits as shown in Table III.

According to Whitby and Britton (*loc. cit.*) any condition which causes dehydration, for example excessive vomiting or diarrhoea, will bring about leucocytosis. Patterson, Mchenry and Crandall (1942) observed a loss of weight in vitamin A deficient rats due to the lack of retention of water, fat and proteins in the animals. If vitamin A is taken as an agent for the retention of water in the body, its deficiency should be expected to be associated with the physiological leucocytosis.

That with the increase of vitamin A level there is a definite tendency for the leucocyte count to decrease is further supported by the differential picture of the leucocytes as discussed below.

None of the subjects in this study showed the type of picture as shown by Abbot *et al.* (*loc. cit.*). On the other hand, the results of this study contradict their findings. They had recognized the clinical manifestations of vitamin A deficiency and it may be that the acute and prolonged vitamin A deficiency is accompanied by leucopenia, as found by them. The subjects of this study were normal persons and the present blood picture may be regarded as the normal reaction of leucocytes to threshold vitamin A deficiency.

Brenner and Roberts (*loc. cit.*) also failed to confirm the results of Abbot *et al.* (*loc. cit.*). The subjects of Brenner and Roberts were so well fed that they did not show any of the signs of subclinical vitamin A deficiency even after seven and a half months of depleted diet. On the other hand the vitamin A level of four out of the six subjects was higher at the end of the depletion period. They, however, observed marked lymphocytosis in their subjects on several occasions during the course of their study. These findings are in accordance with the findings of this study.

2. *Differential count.*—The differential blood picture may be better appreciated from Table III.

As seen from Table III, the defect of the increase of the vitamin level is reflected in each type of the leucocytes. The neutrophils and eosinophils tend to decrease, while the monocytes and lymphocytes tend to increase. The response in monocytes is very distinct. These observations confirm the findings of Brenner and Roberts (*loc. cit.*) as already mentioned. As discussed above, the blood picture supports the inverse relationship between vitamin A and total leucocyte count, both individually and collectively.

3. *Arneth and Schilling count.*—In Table IV, the picture of Arneth count has been shown in relation to vitamin A.

TABLE III.

Vitamin A level I.U. per 100 c.c. plasma.	Number of subjects.	NUMBER OF SUBJECTS WITH NEUTROPHIL PERCENTAGE.			NUMBER OF SUBJECTS WITH LYMPHOCYTE PERCENTAGE.			NUMBER OF SUBJECTS WITH MONOCYTE PERCENTAGE.			NUMBER OF SUBJECTS WITH EOSINOPHIL PERCENTAGE.		
		Above 63.	63-48.	Below 48.	Above 44.	44-30.	Below 30.	Above 6.	6-3.	Below 3.	Above 5.	5-2.	Below 2.
Below normal (110 and below).	11	3	7	1	1	3	7	1	4	9	3	8	0
Normal—													
i. 111-120	14	3	6	5	5	7	2	3	10	1	2	8	4
ii. 121-130	17	1	13	3	5	10	2	5	11	1	3	13	1
iii. 131-140	11	1	9	1	1	9	1	0	10	1	1	9	1
Above normal (above 140).	7	2	3	2	1	4	2	2	5	0	2	4	1

TABLE IV.

Level of vitamin A I.U. per 100 c.c. plasma.	Number of subjects.	NUMBER OF SUBJECTS WITH PERCENTAGES IN ARNETH COUNT IN:												WEIGHTED MEAN.		
		GROUP I.			GROUP II.			GROUP III.			GROUP IV.			Above 2.3.	2.3-1.9.	Below 1.9.
		Above 38.	38-22.	Below 22.	Above 43.	43-35.	Below 35.	Above 30.	30-20.	Below 20.	Above 9.	9-3.	Below 3.			
Below normal (110 and below).	11	3	7	1	1	9	1	1	5	5	0	9	2	0	6	5
Normal—																
i. 111-120	14	4	9	1	1	11	2	3	8	3	1	8	5	2	8	4
ii. 121-130	17	1	14	2	5	9	3	0	14	3	3	12	2	1	15	1
iii. 131-140	11	1	4	6	2	6	3	4	6	1	4	7	1	5	5	1
Above normal (above 140).	7	0	4	3	0	4	3	3	4	0	3	3	1	3	3	1

With the increase in vitamin A level, there is a shift to the right in the Arneth count. The weighted mean also increases. This supports the finding that the neutrophils decrease with the increase in the vitamin content. The fifth group in the Arneth is generally not present.

The complete picture of Schilling's count need not be presented here as the relation of vitamin A with some types of the leucocytes has already been discussed. The types of neutrophils, according to Schilling, in relation to vitamin A are illustrated below (Table V):—

TABLE V.
Schilling's count.

Vitamin A level I.U. per 100 c.c. plasma.	Number of subjects.	NUMBER OF SUBJECTS WITH PERCENTAGE OF STAB. CELLS.			NUMBER OF SUBJECTS WITH PERCENTAGE OF SEGMENTED CELLS.		
		Above 19.	19-11.	Below 11.	Above 45.	45-33.	Below 33.
Below normal (110 and below).	11	5	6	0	2	6	3
Normal—							
i. 111-120 ...	14	4	7	3	4	5	5
ii. 121-130 ...	17	3	11	3	4	9	4
iii. 131-140 ...	11	1	5	5	4	6	1
Above normal (above 140).	7	1	4	2	3	4	0

The juveniles are generally not present in normal persons. Few myelocytes are occasionally found. As shown in Table V there is a shift to the right in Schilling's count with the increase in vitamin A.

4. *The anti-infective properties of vitamin A.*—Bicknell and Prescott (*loc. cit.*) have summarized the clinical literature on the above subject as 'Clinical work on the whole bears out that vitamin A is only of value for increasing resistance to infection when the patients are on a deficient diet'. According to them the general defence mechanisms of the body are not impaired with vitamin A deficiency. The findings of this investigation are in contradiction to these conclusions. As discussed above the vitamin A level affects the blood picture. The leucocytes respond to vitamin A level both differentially and collectively. The relationship exists not only in deficient states but also in optimum levels of the vitamin. In the previous communication, the authors found that the blood sedimentation rate increased with the decrease in vitamin A level. It appears from all this that vitamin A may have definite anti-infective properties. The earlier conclusions are not based on conclusive data, and need revision in the light of new findings.

Part II.

NORMAL WHITE CELL COUNTS IN THE PUNJAB.

The normal hæmogram of white cells in the blood as described in the textbooks, mostly the work of foreign authors, relate to countries with different climates. Data about the white cells and their differential variation for the Punjab has not been reported. Kennedy and Mackay (1936), working in Iraq, have shown that in that country the Cooke polynuclear count is shifted to the left in both natives and Europeans as compared with standards for Britain; this they attribute to climatic conditions. Similarly, some variation may be expected in the total differential number with different climates. Below is given the average normal hæmogram of leucocytes as found in this study and compared with the findings of other workers (Table VI):—

TABLE VI.

Percentages in differential variation.

Authority.	Normal total leucocytes.	Neutrophils.	Lymphocytes.	Mono-cytes.	Eosinophils.	Basophils.
1. Present investigation—						
i. Normal range	6,350–8,650	48–62	30–44	3–6	2–5	0–0.5
ii. Mean value ...	7,510	55	37	4.5	3.5	...
2. Carleton (1943) ...	7,000–8,000	65–70	20–25	3–5	2–4	0–0.5
3. Wright (1940) ...	6,000–8,000	60–70	25–30	5–10	1–4	0–1
4. Best and Taylor (1940)	5,000–9,000	65–70	20–25	5–6	3–4	0–0.5
5. Whitby and Britton (<i>loc. cit.</i>).	5,000–8,000	56–70	21–35	4–8	2–4	0–1
6. Napier and Das Gupta (<i>loc. cit.</i>).	6,542 ± 1,214	62.6	26.9	5.5	4.8	0.2

The following are the hæmograms of Arneth and Schilling (Table VII):—

TABLE VII-a.
Normal Arneth hæmogram.

Authority.	Percentage of groups.				
	I.	II.	III.	IV.	V.
1. Present investigation—					
i. Normal range ...	22-38	35-43	15-25	1-9	0-1
ii. Mean value ...	30	39	25	6	0
2. Carleton (<i>loc. cit.</i>) ...	12	25	46	15	2
3. Best and Taylor (<i>loc. cit.</i>) ...	5	30	45	18	2
4. Whitby and Britton (<i>loc. cit.</i>) ...	10	25	47	16	2
5. Napier and Das Gupta (<i>loc. cit.</i>) ...	37	44	16	3	0

TABLE VII-b.
Schilling's normal hæmogram.

Authority.	Normal total leucocytes.	Basophils.	Eosinophils.	Myelocytes.	Juveniles.	Stab.	Segmented.	Lymphocytes.	Monocytes.
Present investigation	6,350-8,650	0-0.5	2-5	0-0.5	0-1	11-19	33-45	30-44	3-6
Whitby and Britton (<i>loc. cit.</i>).	5,000-8,000	0-1	2-4	0	0-1	3-5	51-67	21-35	4-8

It is evident from Tables VII-a and VII-b that the hæmograms as found in this study are very much different from those given in the textbooks reported by other workers.

SUMMARY.

The blood level of vitamin A in relation to the total number of leucocytes and their differential variation in the blood was studied in 60 normal male students of the K. E. Medical College, Lahore. The average values obtained have been given in Table I.

It has been found that leucocytes, both collectively and differentially, respond to the level of vitamin A in the blood. With the increase in vitamin A content, the total number tends to decrease. Lymphocytes and monocytes tend to increase and neutrophils and eosinophils decrease. There is a shift to the right in the hæmograms of Arneth and Schilling. Vitamin A deficiency brings about physiological leucocytosis.

From the findings of this study it appears that there is some relationship between vitamin A and the general defence mechanisms of the body.

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ACTION OF SOME INDIGENOUS DRUGS ON UTERUS.

A PRELIMINARY NOTE.

BY

R. D. KAPUR, M.D. (Lucknow).

(From the Department of Pharmacology, K. G. Medical College, Lucknow.)

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INTRODUCTION.

A LARGE number of herbs is used in this country by the practitioners of the indigenous system of medicine for the treatment of uterine disorders. As a preliminary step, these drugs in their crude form have been systematically investigated for their action on uterus.

The author has selected the following 22 drugs which are commonly used in India as emmenagogues or ecbolics :—

Botanical name.	Vernacular name.	Part used.
1. <i>Gossypium herbaceum</i> ...	Kapas.	Seeds.
2. <i>Helleborus niger</i> ...	Kali katuki.	Root.
3. <i>Peganum harmala</i> ...	Harmal.	Seeds.
4. <i>Plumbago rosea</i> ...	Lal chitrak.	Root.
5. <i>Rauwolfia serpentina</i> ...	Chota chand.	Root.
6. <i>Rubia cordifolia</i> ...	Manjith.	Root.
7. <i>Nerium odorum</i> ...	Kaner.	Root bark and leaves.
8. <i>Nigella sativa</i> ...	Kulaunji.	Seed.
9. <i>Ruta graveolens</i> ...	Sudap.	The herb.
10. <i>Sesamum indicum</i> ...	Til.	Seed.
11. <i>Anona squamosa</i> ...	Sharifa.	Seed.
12. <i>Boswellia glabra</i> ...	Lobhan.	Gum resin.
13. <i>Cæsalpinhia sappan</i> ...	Patang.	Root and stem.
14. <i>Cardiospermum heliacabum</i> ...	Kanphata.	Root.
15. <i>Eclipta erecta</i> ...	Bongrah mochrand.	Leaves.
16. <i>Euphorbia resinifera</i> ...	Afarbiyun.	Gum.
17. <i>Ficus glomerata</i> ...	Goolar.	The fruit.
18. <i>Mangifera indica</i> ...	Am.	The kernel.
19. <i>Nardostachys jatamansi</i> ...	Balchir.	Rhizome.
20. <i>Parmelia perlata</i> ...	Charela.	Flowers.
21. <i>Pedaliun murex</i> ...	Gokhroo (bara).	Leaves.
22. <i>Woolfordia floribunda</i> ...	Dhanta.	Flowers.

CHEMICAL COMPOSITION.

Before proceeding with the experimental part a brief description of the chemical composition of the above drugs is given below. Out of the 22 drugs, chemical composition of the first ten is mentioned, since the rest proved inert in action on the uterus.

1. *Gossypium herbaceum*.—The seeds have been described to contain a fixed oil (palmitin and olein), albuminoids, lignin and some other nitrogenous substances.

2. *Helleborus niger*.—The roots contain two alkaloids: 'helleborine and helleborein'. The former is insoluble in water and soluble in alcohol and chloroform, while the latter is easily soluble in water, and very sparingly in alcohol and insoluble in ether.

3. *Peganum harmala*.—Dymock *et al.* (1890) mention two principal alkaloids in seeds—'harmaline' and 'harmine'. Another alkaloid 'harmalol', present only in traces, was obtained by Chopra (1933). Besides these three alkaloids the seeds also contain 'peganin', essential oils, saponins and resins.

4. *Plumbago rosea*.—The chief ingredient of the root appears to be an acrid crystalline neutral principle called 'plumbagin'. More recently, Roy and Dutt (1928) have isolated it in a purer form and also solved its chemical structure.

5. *Rauwolfia serpentina* Benth.—The chemistry of this drug has been fairly thoroughly investigated. Greshoff (1890) detected an active principle 'ophioxylon' in the root and he identified it to be of the nature of an alkaloid. Siddiqui and Siddiqui (1931, 1932) and Siddiqui (1939) have worked out its chemistry in detail and isolated five different alkaloids from the root: 'ajmaline, ajmalinine, ajmalicine, serpentine and serpentinine'. They also contain phytosterol, oleic acid, a mixture of unsaturated alcohols, resinous acid and neutral resins.

6. *Rubia cordifolia*.—Nearly all the principles isolated are colouring agents—a red colouring agent 'purpurin', a yellow colouring matter 'munjitin', and another dye 'garnacin'.

7. *Nerium odorum*.—Greenish (1881) was the first person to identify the presence of two bitter glucosides, which he named 'neriodorin and neriodorein', besides a few other ingredients. Lately, Pendse and Dutt (1933) also isolated two amorphous glucosides from the root bark, resembling in properties of the glucosides of Greenish. The root bark also contains small quantities of volatile essential oil, a yellow fixed oil, some tannin and dark colouring matter.

8. *Nigella sativa*.—The chief active principle of the seeds appears to be a toxic glucoside 'melanthin', resembling helleborine. The other ingredients are: (a) a yellowish volatile oil, (b) a fixed oil, (c) metabrin, and small quantities of sugar, albumen, mucilage, organic acids and moisture.

9. *Ruta graveolens*.—The herb yields a small quantity of a volatile oil. This oil of rue consists of about 90 per cent methyl-ketone. A glucoside 'rutin' has also been isolated in very small quantity.

10. *Sesamum indicum*.—The seeds yield a fixed oil, proteids, carbohydrates, mucilage, woody fibre and ash. A crystalline substance called 'sesamin' and a phenol compound have also been obtained from the seeds.

GENERAL PLAN OF INVESTIGATION.

The investigation was divided into three parts: (a) Preliminary experiments were performed on the guinea-pig uterus to find out if the drug produced any action on it. (b) Those which were found to have an action on the guinea-pig uterus were administered to women during puerperium and records of their uterine contractions were taken. (c) Those which, after the clinical trial, were found to have a stimulant action, were next assayed on the guinea-pig uterus, the comparison being made with a standard pituitary extract. Experimental details and tables are omitted to economize space.

EXPERIMENTAL.

SECTION I.—*Experiments on isolated uterus of guinea-pig.*

As stated above, these experiments were carried out to find out which of the drugs under investigation produced any action on the isolated uteri of healthy guinea-pigs (virgin or parous but non-pregnant). The drugs were used in their crude form—an aqueous extract of 1/5 concentration of the various drugs being prepared freshly each time. The action was studied with different concentrations of the drugs ranging between 1/125 and 1/1,000 concentration.

Effect of pH alteration on uterine movements.—It has been observed that an alteration in the pH of the surrounding medium of the uterus sometimes changes its reactivity. Therefore, in the above experiments, to eliminate the possibility of the effect of alteration in the pH on the movements of uterus by the presence of concentrated solutions of the drugs, simultaneous determination of the pH of the solution of different strengths was done with the help of a Hellige colorimeter, using 'universal indicator'. It was observed that in no case the pH varied by more than ± 1 , as compared with Ringer solution as control. Thus, it can be safely concluded that the addition of drug solutions to the surrounding medium of the uterus did not affect the pH to any appreciable degree, and so the actions observed were due to drugs and not by alteration in pH of the solution.

Method of experiment.—The method adopted was the one employed by Dale and Laidlaw (1912). After tying the piece of uterus and allowing time for complete relaxation, a few normal contractions were recorded. The aqueous extract of the drug was next added in such proportions as to make a final dilution of the drug in the uterine bath in the above-noted ranges (1/1,000 to 1/125), and the action produced by the drug was then recorded. In order to determine the site of action of those drugs which produced powerful tonic contraction, paralysis of the parasympathetic nerve-endings was effected by adding atropine solution to the same bath while the drug action was at its climax. In this way action of all the drugs was studied on a number of uteri.

Observations and conclusions.—In tabulating the results of the action of the drugs in the above experiments, note was made of the following points:—

- (a) Rise in height of contraction (contractility).
- (b) Rise in base-line (tonicity).
- (c) Frequency of contractions (rhythmicity).

(d) Effect of atropine on drug action (by paralysing para-sympathetic endings).

The following conclusions were drawn:—

(1) Out of the 22 selected drugs, the first ten produced definite stimulant action on the guinea-pig uterus—increasing the height, tone, and/or frequency of contractions. The action was well marked in higher concentrations of the drugs (see Graph 1, figs. 1–10). The remaining 12 drugs did not show any stimulation.

(2) Almost all the drugs produced tonic rise or spasmodic contraction in 1/125 concentration.

(3) There is no appreciable change in the pH of the solutions by the addition of drugs, and the action produced is, therefore, unaffected by the pH factor.

(4) Atropinization did not relax the tonic contraction.

SECTION II.—*Clinical trials on puerperal patients.*

The first ten drugs, with the exception of *Nerium odorum*, were next tried on women during puerperium (*N. odorum* was not tried therapeutically owing to its reported highly toxic actions). No such work has been reported previously with these drugs.

Method.—Moir's (1935) method of internal hysterography was followed with slight modification, for recording uterine movements. A few salient points of the method are given below:—

(a) *The subject of experiment.*—The persons subjected to the hysterographical measurements were women in puerperium, varying in age from 18 to 40 years. Altogether 35 patients were experimented upon, a different case being taken each time. The time at which they were taken up for the experiment varied from the 6th to the 10th day of puerperium, majority being 7th day cases.

(b) *The drugs.*—The drugs were administered orally in the form of freshly prepared infusion.

(c) *Performance of the test.*—After about 20 minutes from the introduction of the balloon into the uterine cavity, normal movements were recorded for the first half an hour. The drug was then administered to the patient orally and the recording continued for further $2\frac{1}{2}$ hours. In some cases the effect of ergometrine, given orally to the same patient, was recorded after the drug action was over, so as to compare the action of the two.

Observations and conclusions.—The following points were noted in each case:—

(i) Number of confinement.

(ii) Day of puerperium.

(iii) Position and size of uterus.

(iv) Pulse rate—normal and $\frac{1}{2}$ -hourly after drug.

(v) Blood pressure—normal and $\frac{1}{2}$ -hourly after drug.

(vi) Nature of normal movements—(a) time of appearance of movement after onset of recording, (b) height, (c) frequency, and (d) durations of each contraction.

- (vii) Nature of movements after drug—(a) time from administration to commencement of drug action, (b) height, (c) frequency, (d) rise in tone, and (e) duration of each contraction.
- (viii) Action of ergometrine—time of its onset of action, increase in amplitude, frequency and tone, and its action on pulse rate and blood pressure.
- (ix) Toxic or side-effects by drugs on patients.

Each drug was tried in at least three different cases and the observations so made were noted separately for each. The main conclusions, which are given below, are based on the proportion of average increase produced by the drugs in (a) height, (b) rate, and (c) duration of contractions, as compared to normal figures. The conclusions drawn are :—

(1) Definite stimulant action was produced by the first six drugs (*vide infra*) in puerperal patients (*see* Graph 1, figs. 11–13; Graph 2, figs. 14–16).

(2) Of these six drugs, taking into consideration the increase in height as our guide in judging the activity of the drug when compared to normal, *G. herbaceum* and *R. serpentina* are the most active, next in descending order being *H. niger*, *R. cordifolia*, *P. rosea* and *P. harmala*.

(3) None of the drugs produced any toxic side-effects except that in one case there was vomiting after administration of *P. harmala*.

(4) Pulse rate and blood pressure remained unchanged with drugs.

(5) Ergometrine produced its usual action on the uterus but it also increased the pulse rate and blood pressure.

(6) Action of some drugs (*G. herbaceum* and *R. serpentina*) compared very favourably with that of ergometrine on the uterus.

SECTION III.—Experiments for biological assay of drugs.

Under this section experiments were performed to have a comparative quantitative estimation of the oxytocic activity of those drugs which were found to have a stimulant action on the uterus of puerperal patients. The comparison was made by finding out a suitable dose of the drug which would produce contraction of a virgin guinea-pig uterus, nearly equal to that produced by a sub-maximal dose of the standard (*see* Graph 2, figs. 17–22).

Method.—The method adopted was the one described by Burn and Dale (1922) for the standardization of the oxytocic principle of the pituitary extract. The test was performed on three different uteri with each drug and the results were calculated on the average of the three trials.

Observations and conclusions.—The rise in height produced was considered as the criterion for measuring the activity of the drug. The maximum rise produced was measured and, by applying simple mathematical calculation, a dose of the test drug was estimated which would produce the same rise as would be produced by 1 o.u. (oxytocic unit) of the standard. It was concluded from these experiments that *P. harmala* is the most potent in oxytocic activity as compared with the standard, next in descending order being *R. serpentina*, *G. herbaceum*, *P. rosea*, *H. niger* and *R. cordifolia*.

GRAPH 2.

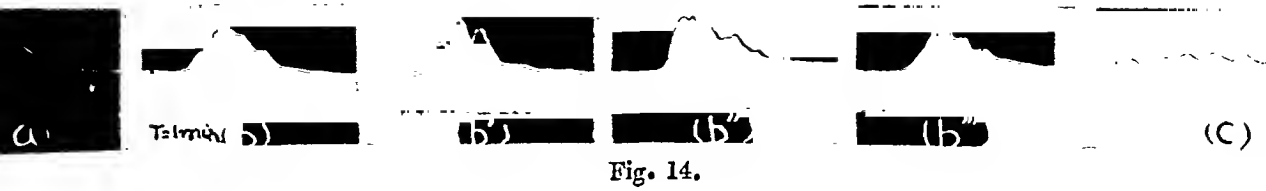


Fig. 14.

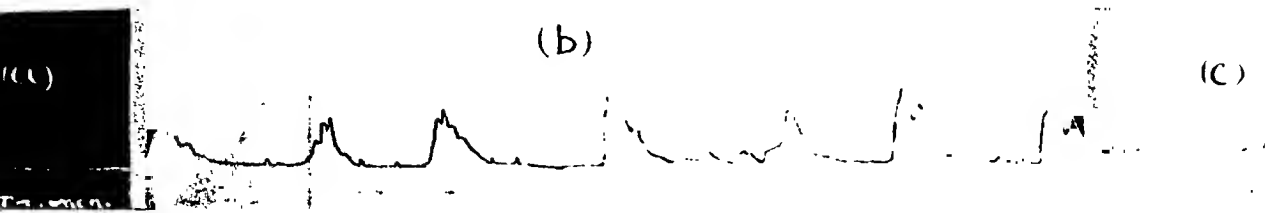


Fig. 15.

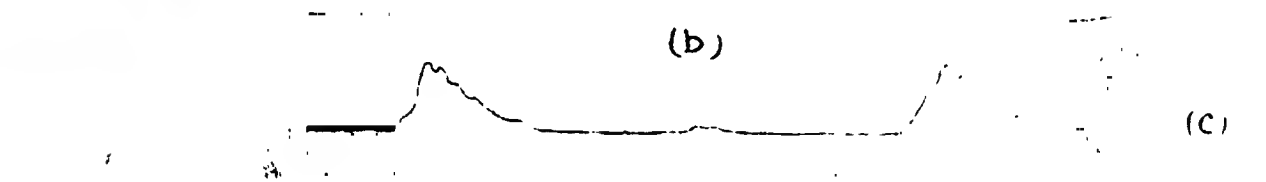


Fig. 16.

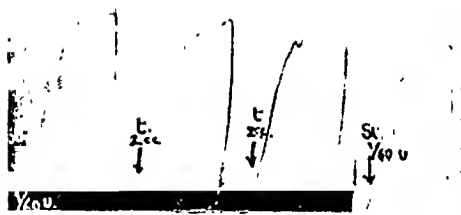


Fig. 17.

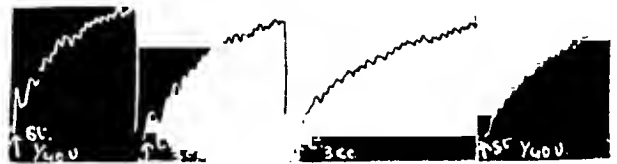


Fig. 18.



Fig. 19.

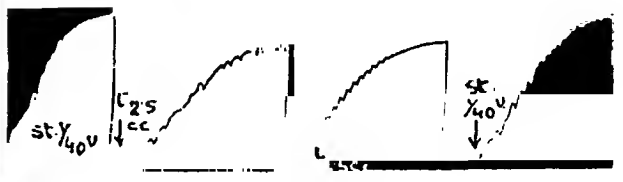


Fig. 20.

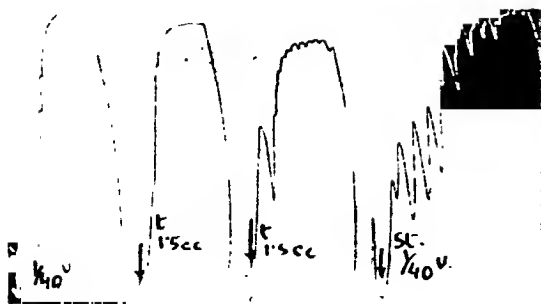


Fig. 21.



Fig. 22.

EXPLANATION OF GRAPH 2.

- Figs. 14-16. Hysterographical tracings of human puerperal uterus.
 „ 17-22. Tracings of assay experiments on guinea-pig virgin uterus.
 (St.=standard drug ; t=test drug.)
-

- Fig. 14. Showing the action of *P. rosea*, (a) normal, (b) 45 minutes after drug, contractions at every 10 minutes (b', b'', b'''), and (c) action of ergometrine.
- „ 15. Showing the action of *R. serpentina*, (a) normal, (b) 1 hour 15 minutes after drug, and (c) action of ergometrine.
- „ 16. Showing the action of *R. cordifolia*, (a) normal, (b) 43 minutes after drug, and (c) action of ergometrine.
- „ 17. Standardization test for *G. herbaceum*.
- „ 18. „ „ „ *P. rosea*.
- „ 19. „ „ „ *P. harmala*.
- „ 20. „ „ „ *R. cordifolia*.
- „ 21. „ „ „ *R. serpentina*.
- „ 22. „ „ „ *H. niger*.

DISCUSSION.

A preliminary study of the action of some indigenous drugs on uterus has been described with a view to elicit information as to which of them have any action on the uterus. Although the general pharmacological action of some of the drugs has been previously reported, as far as the author is aware, no such comparative work of their action on uterus has so far been undertaken collectively.

The action of *P. rosea* on guinea-pig uterus is confirmed. Bhatia and Lal (1933) observed similar action. The author has further noted a stimulant action of the drug on the uterus of patients.

Chopra and Chowdhury (1933) and more recently Gupta and Kahali (1943) obtained a stimulant action of the alkaloid from *R. serpentina* on guinea-pig uterus. Similar is the observation of the author, who also noted that the drug produced stimulant action on the puerperal uterus in patients.

P. harmala was pharmacologically studied by Gunn and Marshall (1920) who observed that the active principles of the drug produce paralysis of the skeletal and plain muscles of the heart. The author has observed stimulant action on the plain muscles of the guinea-pig uterus as well as on the uterus of women in puerperium.

G. herbaceum is a very old remedy reputed for its emmenagogue and galactogogue properties. The drug is reported to have been used in bark form for this, whereas the author has obtained well-marked stimulation with the seeds in both the guinea-pig and the human uterus.

N. odorum, though produced stimulant action on the guinea-pig uterus, has not been tested on patients due to its reported powerful depressant action on the heart.

N. sativa, *R. graveolens* and *S. indicum*, though produced stimulation of the guinea-pig uterus, did not act in patients.

Besides studying the action on the guinea-pig and human uterus, an effort has been made to compare the oxytocic activity of the active (first six) drugs with a standard drug 'oxytocin'. As a result of these assay experiments, *P. harmala*, *R. serpentina* and *G. herbaceum* proved to be more active than others—an observation which coincides with the results achieved in therapeutic trials.

SUMMARY.

1. Pharmacological action of 22 indigenous drugs reported to be useful in uterine disorders has been reported in this paper.

2. Ten of the selected drugs (*G. herbaceum*, *H. niger*, *P. harmala*, *P. rosea*, *R. serpentina*, *R. cordifolia*, *N. odorum*, *N. sativa*, *R. graveolens* and *S. indicum*) produced stimulant action on the guinea-pig isolated uterus.

3. Six of them (*G. herbaceum*, *H. niger*, *P. harmala*, *P. rosea*, *R. serpentina* and *R. cordifolia*) were found to stimulate the uterus of women in puerperium.

4. The oxytocic activity of these six drugs was biologically compared with oxytocin, and in descending order of their activity the drugs could be arranged as *P. harmala*, *R. serpentina*, *G. herbaceum*, *P. rosea*, *H. niger* and *R. cordifolia*.

The author is highly indebted to Dr. B. B. Bhatia, M.D., M.R.C.P. (Lond.), Head of the Department of Pharmacology, and to Dr. (Miss) G. H. Marchant, M.D., F.R.C.S., M.C.O.G., Head of the Department of Obstetrics and Gynæcology, K. G. Medical College, Lucknow, for giving personal help and all the facilities of their respective departments to the author in the completion of the above work.

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PHARMACOLOGICAL ACTION OF ALKALOIDS OF *R. SERPENTINA* BENTH.

Part II.

TOTAL ALKALOIDAL EXTRACTS OF BIHAR AND DEHRA DUN VARIETIES.

BY

R. D. KAPUR, M.D. (Lucknow).

(From the Department of Pharmacology, K. G. Medical College, Lucknow.)

[Received for publication, September 12, 1947.]

IN a previous paper Bhatia and Kapur (1944) published results of their observations on the action of two newly isolated alkaloids extracted from the Dehra Dun variety of *R. serpentina* Benth. Since the action of the various alkaloids isolated from the Bihar and Dehra Dun varieties of the plant varies, a comparative study of their total alkaloidal extracts was made. As the findings appeared to be different in some respects from those of Gupta and Kahali (1943), who had previously carried out similar observations in respect of total alkaloidal extracts isolated from the Bengal, Bihar and Dehra Dun varieties of the plant, it is considered necessary that these should be reported here.

Two alkaloidal extracts were received through the courtesy of Dr. S. Siddiqui, Ph.D., Assistant Director of the Board of Scientific & Industrial Research, New Delhi. These represent the total alkaloidal contents of the roots as they exist in nature and are provisionally named as 'Neo-ajmaline totalate' (abbrev. N.A.T.) and 'Ajmaline totalate' (abbrev. A.T.) isolated from Dehra Dun and Bihar plants, respectively. Both extracts are highly soluble in water. N.A.T. gives a clear brownish solution, while A.T. gives a slightly translucent bright-yellow solution. A 1 per cent aqueous solution was generally used in all the experiments; it keeps well for about 48 hours in unsterile condition.

Results of this study are presented below :—

Systems.	Ajmaline totalate.	Neo-ajmaline totalate.
Local action—		
(On the eye) ...	Instillation of 1 per cent solution in the eyes of rabbits and guinea-pigs produces slight irritation of the cornea but no action on the pupils.	
(On the skin) ...	In frogs, redness within 5 to 10 minutes of injection over their whole ventral surface. In some cases even blood-stained fluid exuded through the skin. In white rats, necrosis and ulceration of superficial tissues at the site of injection.	
Toxicity—		
(a) M. l. d. in frogs ...	0.13 mg./g. body-weight.	0.17 mg./g. body-weight.
(b) Av. l. d. in mice ...	0.175 mg./g. body-weight.	0.225 mg./g. body-weight.
Central nervous system—		
(a) Frogs ...	Signs of irritation, excitement and restlessness in the beginning, followed by depression of motor activity and reflexes. In spinal frogs, action of strychnine was diminished and after injection of the extracts.	
(b) White rats ...	Immediately after injection restlessness due to local irritation. Later, there was depression of motor activity and reflexes. There was no hypnosis. Respiration was quick in the beginning but later decreased.	
Frog's heart and cardiac output.	Heart is depressed and output diminished. A-V block is produced, since there is no alteration in action on injection of atropine. The heart does not regain normal activity for a long time even after change of Ringer solution.	
Isolated guinea-pig's heart	Depression of systolic contraction, and gradual dilatation of heart, tending to stop in diastole.	
Blood vessels ...	Vaso-constriction.	Vaso-constriction.
Organ volume ...	Spleen volume increased in cats but decreased in dogs. Intestinal volume increased in cats and dogs both. There is visible increase in intestinal peristaltic waves <i>in situ</i> .	
Carotid pressure, normal and raised.	Decreased in all—intact, spinal, decerebrate animals and in artificially raised condition. In equal doses, ajmaline totalate has more powerful action than neo-ajmaline totalate.	
Tracheal respiration ...	At first the rate and depth are slightly increased but later decreased.	
Isolated uterus ...	Amplitude and tone decreases. Recovery to normal after a long time. Addition of CaCl_2 during drug depression produces very feeble action, but pilocarpine brings normal response—this shows that the extracts act directly on muscles.	
Isolated intestine ...	Decrease in movements, amplitude and tone; recovery to normal after a long time. Action seems on muscles directly. Addition of CaCl_2 produces no change, but pilocarpine induces its normal action when added to the same bath as contained in the extract.	

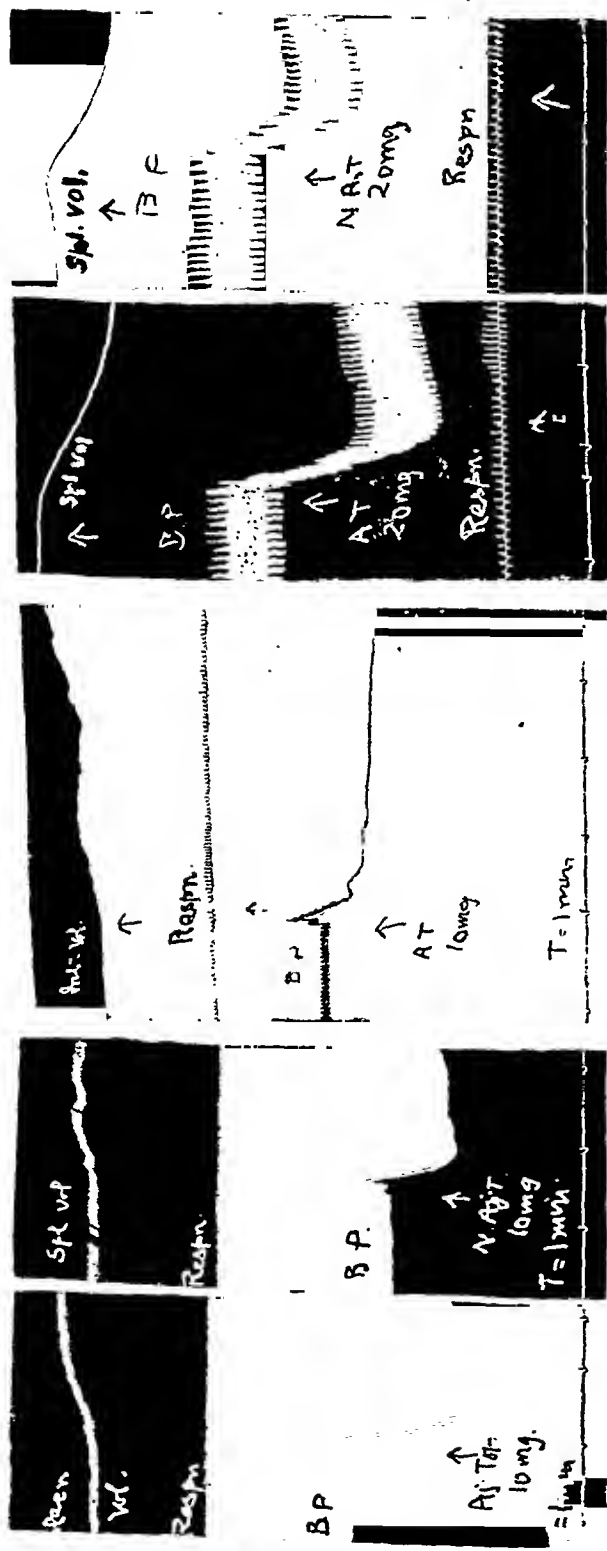


Fig. 1. (a)

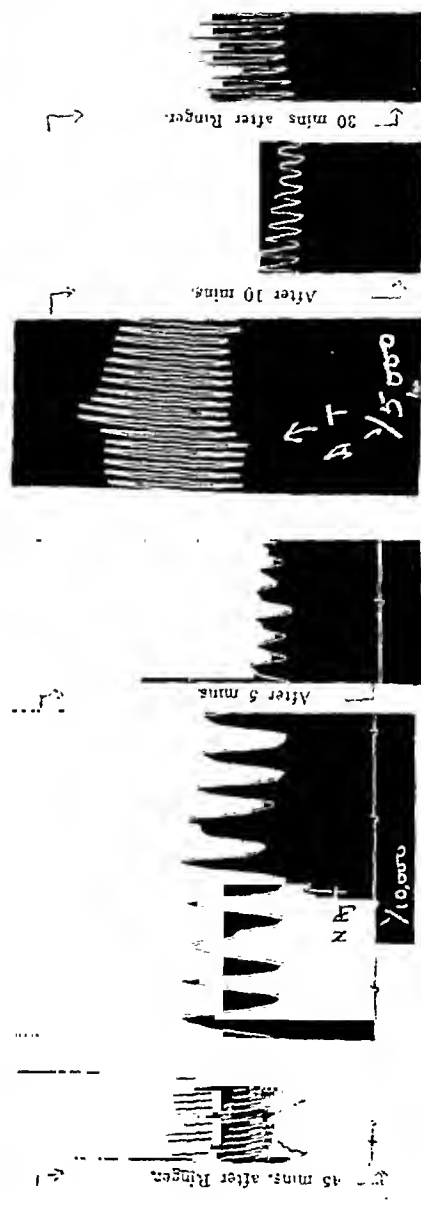
(b)

(c)

(d)

(e)

Fig. 1.—(a) and (b)—Cat's blood pressure, respiration and spleen volume, (c) cat's blood pressure, respiration and intestinal volume, (d) and (e) dog's blood pressure, respiration and spleen volume.



(a)

(b)

(c)

(d)

(e)

(f)

Fig. 2. (a)

(b)

(c)

(d)

(e)

(f)

Fig. 2.—Action on frog's heart and cardio output. At A.T. atropine totalato 1/5,000 is perfused; at Atr. atropine 1 c.c. 1/1,000 injected. (Upper tracing—ht. record. Lower tracing—cardio output, each downstroke 1.8 c.c.)

DISCUSSION.

From a perusal of the above results, it will be seen that the total alkaloidal extracts from the Bihar and Dehra Dun plants are very similar in their actions on the various systems, the difference being one of degree. The chief actions of the total alkaloids are also on the same systems as noted with pure alkaloids, viz. on the central nervous system and the cardiovascular system. While the action of the extracts is very much similar to alkaloids, they are safer to administer for therapeutic purposes, since there is a good margin between the therapeutic dose and the lethal dose in extracts.

The action of the two alkaloids on the nervous system of frogs is identical, but in white rats, the Bihar extract (ajmaline totalate) produces depression. No hypnotic action was seen in mice with either extract as has been reported by Gupta and Kahali (*loc. cit.*).

On the cardiovascular system the action of the two extracts is very similar. The heart is depressed and its output diminished. Carotid pressure is lowered in all conditions, there being only a difference of degree. Chopra *et al.* (1943) mention stimulation of heart, but Gupta and Kahali (*loc. cit.*) and the present author observed depression. Gupta and Kahali's observations tally very much with those of the author, but they obtained rise in carotid pressure in spinal cats, whereas the author and Chopra *et al.* (*loc. cit.*) recorded a fall. This difference in action is not easily explainable, except by assuming that the various samples of total alkaloidal principles varied in their ingredients. The fall in carotid pressure seems to be partly due to depression of the heart and partly as a result of dilatation of the spleen volume. The peculiar behaviour of the spleen volume in dogs, i.e. contraction, cannot easily fit in the picture here, and does not lend support to the idea of fall in pressure being partly produced by splenic dilatation.

There is a difference in action on blood vessels as observed by the author and by Gupta and Kahali (*loc. cit.*). The latter report vaso-dilatation, whereas the author obtained vaso-constriction following the Loewen-Trendelenburg method.

Lastly, the author noted depression of the isolated uterus and intestines of guinea-pigs and rabbits, whereas Gupta and Kahali report relaxation of intestine and stimulation of uterus. The action seems to be on the muscles directly since addition of calcium chloride fails to induce its normal action and pilocarpine produces its usual stimulation. The only possible explanation about the differences in the observations made herein and those of others may be due to difference in ingredients of extracts used, as pointed out above.

SUMMARY.

1. The pharmacological action of the total alkaloidal extracts from the Bihar and Dehra Dun roots of *R. serpentina* is described.
2. Both the extracts produce fall in carotid pressure, Bihar one having somewhat more powerful action.
3. Heart is also depressed by both the total extracts nearly to the same extent.

4. The two extracts produce depression on the central nervous system, but no hypnosis.

5. Uterine and intestinal musculature are depressed by the two extracts in the same way.

6. The Bihar extract is more toxic than the Dehra Dun variety as determined by lethality tests.

The author is indebted to Dr. S. Siddiqui, ph.D., Assistant Director of the Board of Scientific & Industrial Research, New Delhi, for kindly supplying the alkaloidal extracts. The work was done by a grant of Fellowship by the Indian Research Fund Association, New Delhi.

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ON THE OXIDATION OF ACETOACETIC ACID IN
PRESENCE OF NORMAL AND DIABETIC
PLASMA AS WELL AS OTHER
KETOLYTIC COMPOUNDS.

STUDIES *IN VITRO* AND *IN VIVO*.

BY

M. C. NATH, D.Sc.,
Chitnavis Professor of Biochemistry,
Department of Biochemistry, Nagpur University,

AND

A. H. M. HABIBUL ISLAM, M.Sc.,
Physiological Section, Department of Chemistry, Dacca University.

[Received for publication, October 13, 1947.]

It was first pointed out by Hirschfeld (1895) that keto bodies appear in the urine when the catabolhydrate carbolized within the system is small and Dakin (1942) and Nath and Brahmachari (1944, 1946) observe that these are responsible to a great extent for the onset of the diabetic troubles. Geelmuyden (1904) was the first to point out that a type of definite chemical reaction might take place between some of the acetone bodies or their precursors and glucose or some of its derivatives.

Somogyi (1942) found that in healthy individuals a measurable decrease of the ketonaemic level takes place after the administration of glucose, which can be explained by assuming that some sort of chemical reaction takes place in the animal system between glucose or its derivatives and the keto bodies or some of their derivatives or precursors as suggested by Shaffer (1921) and observed by West (1925) and Moore, Erlanger and West (1936) (*in vitro* experiments), thus causing a reduction of the total keto bodies in the blood.

Roy (1942) observed that intravenous injection of glucose alone can bring about relief only in 50 per cent cases of diabetic patients. Somogyi and Welselbaum (1942) noticed, on the other hand, that in diabetics as well as in sub-totally

de-pancreatized dogs there is rather an increase of the existing ketonæmia after glucose feeding, rather than any indication of its decreasing at all. It can, therefore, be suggested that proper relief for a diabetic having ketonæmia is possible only when one takes an additional recourse to some such compounds which may have the property of acting as ketolytic agents when administered into the system. If the high concentration of keto bodies can be relieved, the high concentration of glucose also cannot but come down automatically in course of time. It can thus be assumed that the chemical combination of glucose or its derivatives with the keto bodies, which is supposed to take place in the normal individuals, may be wanting in a diabetic system due to the deficiency of some factor not yet known.

That excess concentration of glucose in the blood has no damaging influence on the β -cells of the islands of Langerhans, has been shown clearly by Houssay *et al.* (1942). All these confirmed the idea of Roy and Mukherjee (1939) that increase in hyperglycæmia is a result of some physiological response.

Hence one has to think about reducing those constituents, which are responsible for causing hyperglycæmia, rather than of reducing blood-sugar, at the outset, in diabetes and to find out a substance or substances which can cause ketolysis and can thus be effective in doing away with the cause of the disease and not the symptoms alone.

This may be recorded with interest that amellin, the new anti-diabetic principle isolated by Nath (1941-42), has been found by Nath, Chakraborty and Brahmachari (1945) to relieve ketonæmia in diabetes when administered orally, in addition to its other physiological significanees, e.g. reduction of cholesterolæmia (Nath and Chowdhury, 1943a), decrease of high inorganic phosphate in the blood (Nath and Chowdhury, 1943b), reduction of non-protein nitrogen level of blood and urine (Nath and Chowdhury, 1945), increase in hæmatopoietic activity (Nath, Chakraborty and Banerjee, 1943) and ultimate reduction of hyperglycæmia (Nath and Banerjee, 1943).

Attempts have, therefore, been made to see if this new compound possesses any ketolytic effect within the normal animals and whether the *in vitro* oxidation of keto acids with some oxidizing agent can be accelerated by addition of either normal blood or amellin or both as is expected from this hypothesis.

EXPERIMENTAL.

A solution of the alkaline Na salt of acetoacetic acid was made according to the method of Shaffer (*loc. cit.*) and the acetone content of 10 c.c. of the solution was found out according to the method of Behre and Benedict (1940) modified by Chakraborty and Nath (1947).

Method of procedure.—Ten c.c. of the prepared alkaline Na salt of acetoacetic acid solution were taken in a centrifuging tube to which were added 22 c.c. of water and 3 c.c. of 25 per cent CuSO_4 solution so that the volume was made up to 35 c.c. and then sufficient lime added until the solution was alkaline to litmus paper. It was then centrifuged and 21 c.c. of the supernatant fluid representing 6 c.c. of the solution were transferred by a pipette in the distilling flask. To the solution was added sufficient water to bring the volume to about 50 c.c.; 8 c.c. of 20N. H_2SO_4 were then added to the solution and heating begun. When the solution just

began to boil 2 c.c. of 2.5 per cent potassium dichromate, that was kept in readiness in the bent funnel, were added and distillation continued until 25 c.c. of the distillate were obtained in about 40 minutes; from this solution acetone was estimated colorimetrically according to the method described below:—

Final estimation of acetone.—0.1 c.c. salicylaldehyde was taken in each of the two test-tubes marked 10 c.c. To one were added 2 c.c. of distillate and to the other 2 c.c. of the standard acetone solution. Now, to each of these test-tubes, 1.5 c.c. of a saturated solution of caustic potash were introduced and the reaction allowed to go on for about 18 minutes with frequent stirring by means of a glass-rod. The precipitates formed in the test-tubes were then dissolved by addition of water to make the volumes up to 10 c.c. in each and compared in a colorimeter. Thus, the amount of acetone in 2 c.c. of the distillate was found out, and from that the total quantity of acetone in 10 c.c. of the original solution was calculated.

RESULTS.

In the actual experiment to find out the effect of a certain compound on the oxidation of acetoacetic acid four reaction vessels were used. To each of these vessels were taken 10 c.c. of the prepared alkaline Na salt of the keto acid and the measured amount of hydrogen peroxide and other things including water were added so as to make the volume up to 35 c.c. The temperature of all the solutions was maintained at 38°C. before the addition. The method of procedure to find out acetone bodies in the reaction vessel was followed immediately after the addition of different reagents from the vessel No. 1 in order to get the initial value and the estimation in other vessels (Nos. 2, 3 and 4) was made at intervals of 2, 6 and 24 hours from the beginning of each reaction.

Results are given in Tables I to VII and some are represented graphically (see Graphs 1 to 4).

TABLE I.

Oxidation of acetoacetic acid by H_2O_2 at different alkalinity at 38°C.

Solution number.	SODIUM ACETOACETATE + H_2O_2 + NaOH.			ACETONE IN MG. AFTER DISTILLATION.			
	Na salt.	H_2O_2 in c.c.	Final strength of NaOH.	Initially.	After 2 hours.	After 6 hours.	After 24 hours.
I	10 c.c. = 10.637 mg. acetone.	10	1.0N	10.21	9.75	8.66	4.37
II	..	10	0.5N	10.28	9.75	9.18	5.70
III	..	10	0.25N	10.634	10.44	9.92	...
IV	..	10	0	10.63	10.6	10.6	...

Thus, it is obvious that sodium acetoacetate is oxidized very slowly in 0.25N. NaOH *in vitro*. As an alkalinity even like this does not exist in the body it cannot be supposed that *in vitro* and *in vivo* reactions are identical.

As the presence of glucose was found by Shaffer (*loc. cit.*) to cause greater oxidation, some observations were also made with 100 mg. of glucose at different alkalinity. The mixture before estimation was de-saccharified with 25 per cent CuSO_4 and lime powder according to the usual method.

TABLE II.

Influence of glucose on the oxidation of acetoacetic acid by H_2O_2 in presence of alkali at 38°C.

Solution number.	10 C.C. OF Na ACETOACETATE (= 10.637 MG. ACETONE) + 100 MG. OF GLUCOSE AT DIFFERENT ALKALINITY.		ACETONE IN MG. AFTER DISTILLATION.			
	H_2O_2 in c.c.	Final strength of NaOH.	Initially.	After 2 hours.	After 6 hours.	After 24 hours.
I	10	1.0N	10.32	8.13	3.98	1.80
II	10	0.5N	10.32	8.40	4.01	1.87
III	10	0.25N	10.43	9.51	5.1	2.05
IV	0	0.5N	10.43	10.32	...	10.02

Ketolytic effect of normal plasma.—When it is found that glucose alone cannot cause any appreciable change of acetoacetic acid even in 24 hours in 0.5N. NaOH solution and there is but little chance of any presence of H_2O_2 in the living tissues, it was thought desirable to see if the presence of normal plasma alone has any influence towards oxidation of the keto acid. Table III shows the results.

For this experiment normal human plasma was used. Before proceeding with the estimation of acetone, the solution after addition of the plasma was de-proteinized according to the method of Welchselbaum and Somogyi (1941) and de-saccharified as before.

TABLE III.

Effect of normal plasma on the oxidation of acetoacetic acid with and without H_2O_2 in presence of alkali at $38^\circ C$.

Solution number.	SODIUM ACETOACETATE TAKEN 10 C.C. = 10.637 MG. OF ACETONE.			ACETONE IN MG. AFTER DISTILLATION.			
	Plasma in c.c.	H_2O_2 in c.c.	Final strength of NaOH.	Initially.	After 1 hour.	After 2 hours.	After 24 hours.
I	1.5	0	1.0N	8.77	4.56	3.56	3.08
II	1.5	0	0.5N	8.63	4.07	3.78	3.56
III	1.5	0	0.25N	10.36	5.7	4.2	3.56
IV	1.5	10	1.0N	7.6	1.85	1.42	0.95
V	1.5	10	0.5N	8.14	2.10	1.62	1.3
VI	1.5	10	0.25N	9.5	2.72	1.71	1.3
VII	1.5	10	1.0N	10.21	9.75	8.66	4.87

It is very interesting to record that acetoacetic acid is oxidized to a great extent in presence of normal plasma alone even without H_2O_2 . This gives us a definite clue as to how the physiological oxidation takes place within the system even though Green says 'It is yet to be demonstrated that H_2O_2 occurs to any appreciable extent under physiological condition', as quoted by Harrow (1945).

Amellin, which has recently been found by Nath, Chakraborty and Brahmachari (*loc. cit.*) to cause reduction of acetone bodies in the blood of diabetics, was then used in order to see if it had any effect on the oxidation of acetoacetic acid *in vitro*. Table IV shows the results.

In studying the ketolytic action of amellin the mixture of the solutions was de-proteinized before de-saccharification and estimation of acetone.

TABLE IV.

Effect of amellin on the oxidation of acetoacetic acid in presence of H_2O_2 in alkaline medium at $38^\circ C$.

Solution number.	Na ACETOACETATE TAKEN 10 C.C. = 10.637 MG. OF ACETONE.			ACETONE IN MG. AFTER DISTILLATION.			
	Amellin in mg.	H_2O_2 in c.c.	Final strength of NaOH.	Initially.	After 2 hours.	After 6 hours.	After 24 hours.
I	50	10	1.0N	9.51	4.10	3.0	1.61
II	50	10	0.5N	9.42	4.31	3.1	1.4
III	50	10	0.25N	10.1	4.3	4.0	1.93
IV	50	0	1.0N	11.53	11.50	11.40	11.34

It is thus evident that amellin which has been found by Nath *et al.* to cause great relief in diabetes is not an oxidant itself like the normal plasma but its presence along with other oxidizing agent such as H_2O_2 accelerates the process to a very great extent.

Attempts were next made to find if the presence of glucose can exert any additional influence in the process of oxidation of acetone bodies by H_2O_2 while mixed with amellin and whether amellin and glucose can cause oxidation of acetoacetic acid without any oxidizing agent. Table V shows the results.

TABLE V.

Ketolytic action of amellin and glucose with and without H_2O_2 in alkaline medium at $38^\circ C$.

Solution number.	SODIUM ACETOACETATE USED 10 C.C. = 10.637 MG. OF ACETONE; ALKALINITY = 0.5N.			ACETONE IN MG. AFTER DISTILLATION.			
	Glucose in mg.	Amellin in mg.	H_2O_2 .	Initially.	After 2 hours.	After 6 hours.	After 24 hours.
I	100	50	10	10.55	5.73	2.60	1.30
II	100	50	0	11.40	11.26	10.9	10.32
III (from Table II)	100	0	0	10.43	10.32	...	10.02
IV (from Table II)	100	0	10	10.32	8.40	4.01	1.87
V (from Table I)	0	0	10	10.28	9.75	9.18	5.70

It is thus seen that there is little additional effect of glucose on the ketolytic influence of amellin + H_2O_2 , while amellin + H_2O_2 has been found to possess greater ketolytic influence than glucose + H_2O_2 . After having an idea about the ketolytic influence of normal plasma and that of amellin which is highly beneficial in diabetes mellitus, it was thought advisable to see whether in case of severe attack of diabetes, plasma shows the same ketolytic effect as in normal cases or it differs, and if so to what extent? Table VI shows the experimental findings.

TABLE VI.

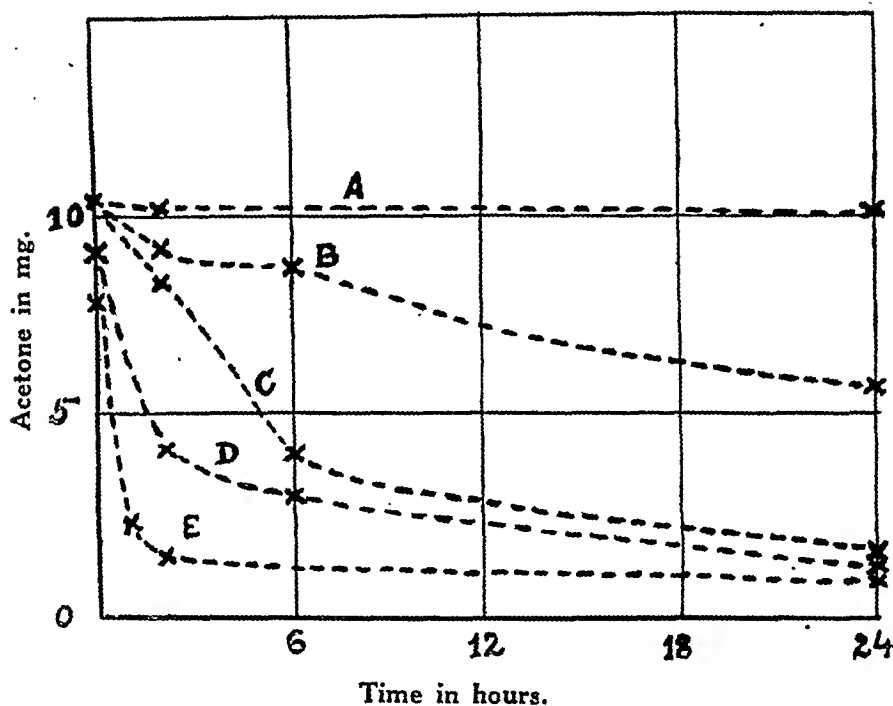
Ketolytic effect of plasma from diabetic patients with and without H_2O_2 in alkaline medium at $38^\circ C$.

Solution number.	SODIUM ACETOACETATE TAKEN 10 C.C. = 10.637 MG. OF ACETONE.			ACETONE IN MG. AFTER DISTILLATION.			
	Diabetic plasma in c.c.	H_2O_2 in c.c.	Final strength of NaOH.	Initially.	After 2 hours.	After 6 hours.	After 24 hours.
I	1.5	0	0.5N	10.20	9.6	8.7	8.1
II	1.5	10	0.5N	9.8	6.7	2.0	1.2

The results have also been represented graphically to make a comparative study of the degree of ketolysis caused by different substances *in vitro* (see Graphs 1 to 3).

It has been pointed out by Koehler, Windsor and Hill (1941) that neither glucose nor insulin has any direct ketolytic effect *in vivo*. Hence some observations on the effect of amellin *in vivo* (in normal animals) towards ketolysis was

GRAPH I.



Strength of NaOH 0.5N.

- A. Glucose alone.
- B. Hydrogen peroxide alone.
- C. Glucose + hydrogen peroxide.
- D. Amellin + hydrogen peroxide.
- E. Normal plasma + hydrogen peroxide.

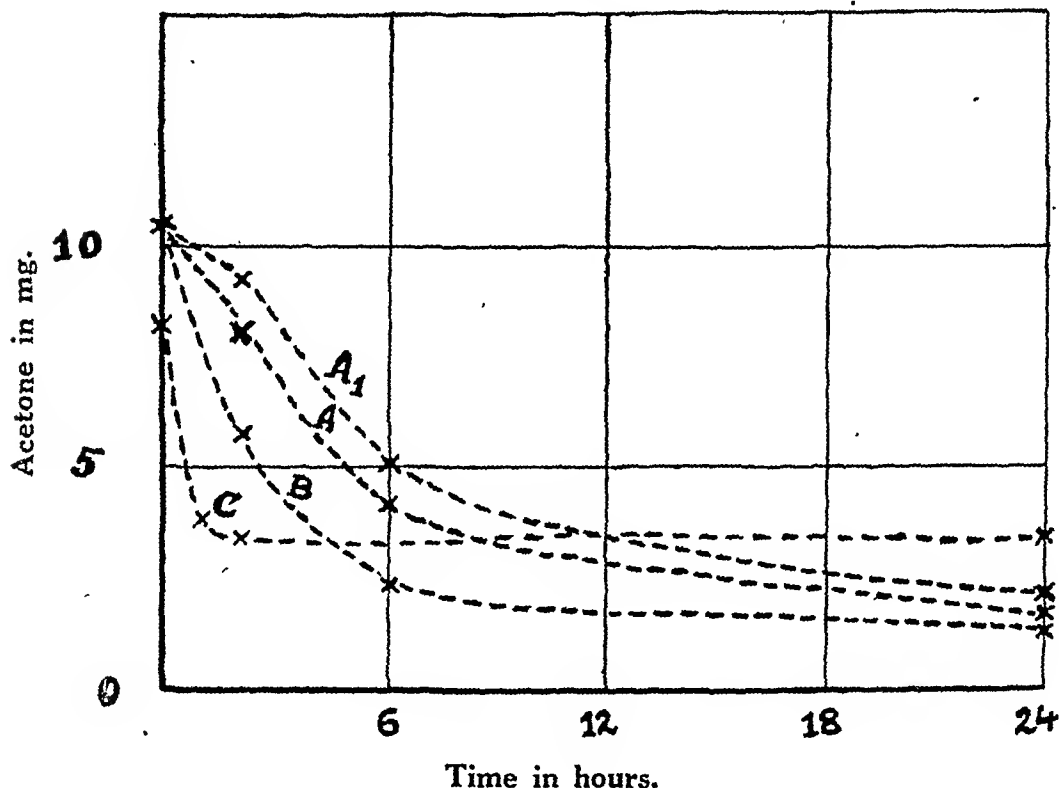
thought to be absolutely essential. The following experiments were, therefore, made:

Some observations in vivo.—For this experiment three healthy rabbits were made to fast for about 24 hours. Three c.c. of blood from each were collected.

and acetone bodies were estimated according to the usual method but no measurable quantity was detected in any of these rabbits.

As it is very difficult to collect 3 c.c. of blood from a rabbit each time, for 3 or 4 times a day, 1 c.c. of blood was collected from each of the three experimental animals in each set and the acetone value which was estimated with this mixed

GRAPH 2.



Strength of NaOH 0.5N.

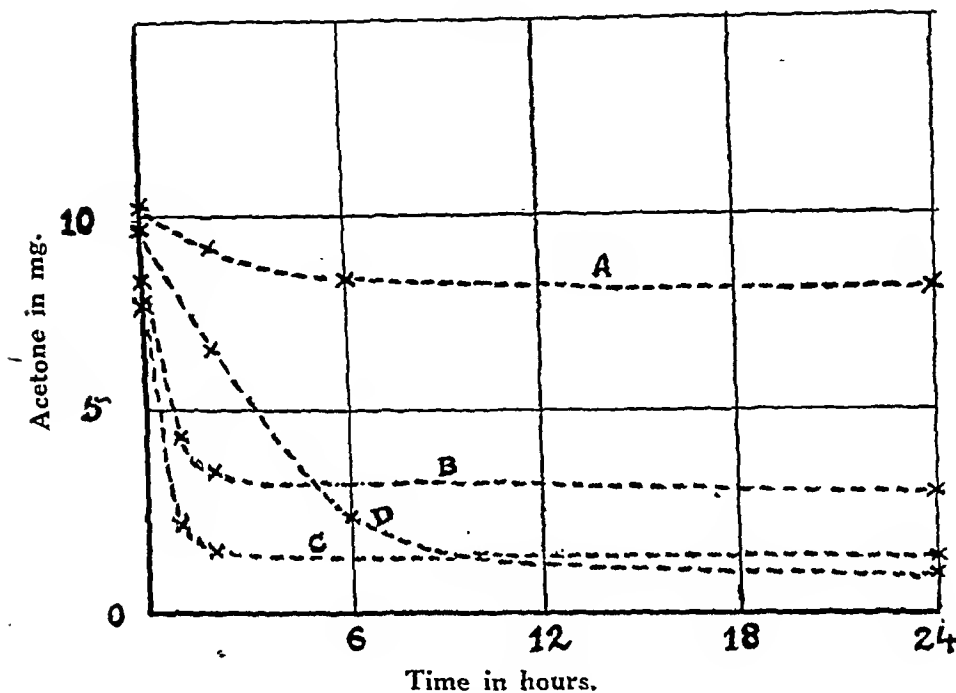
- A. Glucose + hydrogen peroxide.
- A₁. Glucose + hydrogen peroxide (0.25N. NaOH).
- B. Amellin + glucose + hydrogen peroxide.
- C. Normal plasma alone.

blood was taken as the mean value for the three animals. The results are shown below :

(a) Three healthy rabbits, each weighing about 2 kg., were now made to fast for 24 hours and 300 mg. of acetoacetic ester were injected to each. After some definite intervals 3 c.c. of blood were collected from the three animals (1 c.c. from each) and the mean value of acetone per 100 c.c. was calculated.

(b) In another set of experiments simultaneous injections of 300 mg. of acetoacetic ester and 200 mg. of amellin were given to three healthy fasting rabbits

GRAPH 3.



Strength of NaOH 0.5N.

- A. Diabetic plasma.
- B. Normal plasma.
- C. Normal plasma + hydrogen peroxide.
- D. Diabetic plasma + hydrogen peroxide.

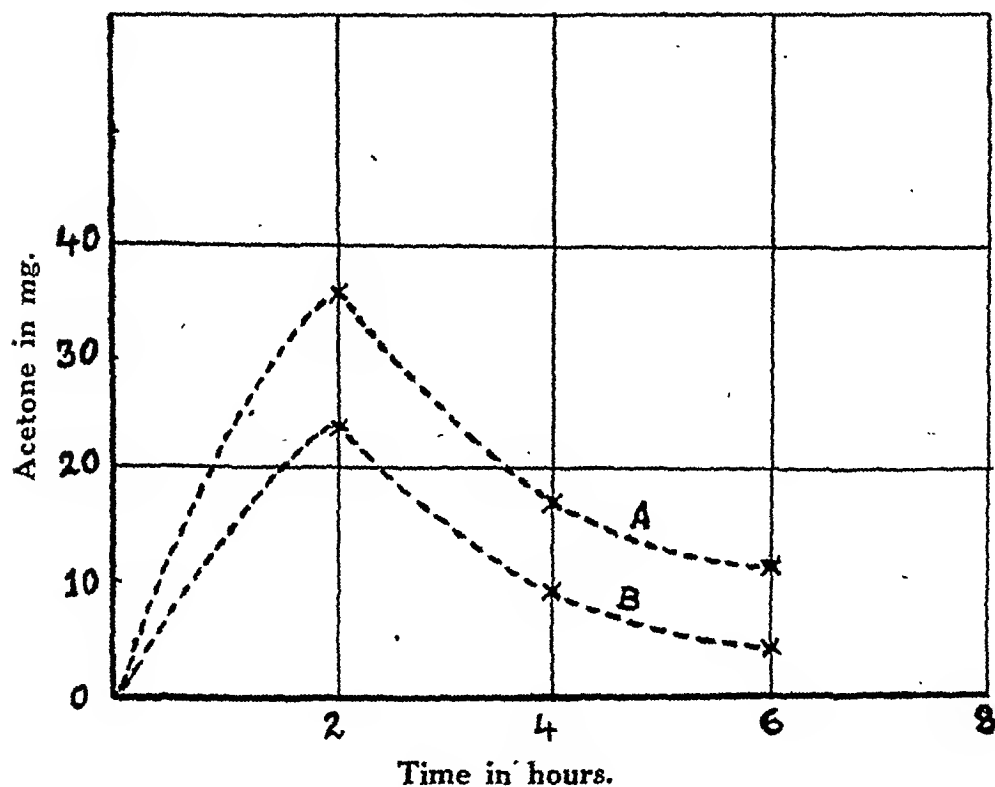
of about 2 kg. body-weight and the acetone contents of their blood estimated as before at regular intervals of time.

TABLE VII.

Experiment number.	Number of animals in each set.	SUBSTANCE INJECTED.		MEAN VALUE OF ACETONE IN MG. PER 100 C.C. OF BLOOD.			
		Acetoacetic ester in mg.	Amellin in mg.	Initially.	After 2 hours.	After 4 hours.	After 6 hours.
A	3	300	0	Trace	35.5	17.6	11.2
B	3	300	200	"	24.0	9.2	4.6

The results are also given in Graph 4, which shows how amellin can also act in a far better way than glucose or insulin (Koehler *et al.*, *loc. cit.*) in causing oxidation of acetoacetic acid and thus give satisfactory and confirmatory explanation how this substance brings about relief in ketonæmia, acidosis, adiposity and excess concentration of lipoids and high protein nitrogen in the blood as previously observed by Nath *et al.* (*loc. cit.*).

GRAPH 4.



Strength of NaOH 0.5N.

- A. Without amellin.
- B. With amellin.

DISCUSSION.

It will be seen from Table I that while acetoacetic acid undergoes oxidation by means of hydrogen peroxide and glucose to the extent of about 20 per cent within a period of two hours' time the same substance gets oxidized to the extent of more than 50 per cent by hydrogen peroxide in presence of amellin, other conditions remaining identical.

It will also be seen from Tables II and IV that though hydrogen peroxide alone can oxidize acetoacetic acid very slowly at an alkalinity of 0.25N. NaOH the rate is greatly enhanced by the addition of amellin.

These clearly show the enormous influence of amellin in bringing about ketolysis *in vitro* in presence of an oxidizing agent such as hydrogen peroxide.

The nature of curves with glucose and H_2O_2 when compared with that with amellin, glucose and H_2O_2 (Graph 2) and also with plasma and H_2O_2 is also of great significance. In case of glucose + H_2O_2 there is observed an initial inhibition and the reaction is accelerated only after some time is allowed to lapse, thus indicating that glucose requires some sort of change before it can take part in the combination or condensation with the keto bodies so as to give rise to some unstable and readily oxidizable compound. In case of amellin with glucose and hydrogen peroxide or with normal plasma and glucose or plasma alone the reaction is found to take place spontaneously just after addition of these reagents to the sodium acetoacetate solution, thus showing how ketolysis takes place in the blood under normal condition and how amellin is helpful in the process.

Graph 3 shows that though the normal plasma brings about rapid oxidation of the keto compounds even in absence of hydrogen peroxide, the diabetic plasma, which is almost ineffective as such, cannot become so effective even in presence of H_2O_2 within the first few hours of reaction. The nature of the curves showing the reaction with normal and diabetic plasma seems to be of entirely different nature. This shows that the diabetic plasma is not only deficient in its contents of oxidizing agents but also of other principles helping the reaction. It will also be seen from Graphs 1 and 2 that though glucose and hydrogen peroxide are far more effective in causing oxidation of acetoacetates than hydrogen peroxide alone, the activity is greatly increased by addition of amellin, which may therefore serve the purpose of making up the deficiency in the blood of a person suffering from diabetic troubles.

Though it has not yet been shown definitely that H_2O_2 occurs in any appreciable extent in the normal blood it has been definitely known that the peroxidases occur in various plant and animal tissues, which catalyse the oxidation of a number of compounds, such as epinephrin, tyrosin, etc., only in presence of hydrogen peroxide or certain other organic peroxides.

These observations also help to a great extent to reconcile the findings with amellin *in vivo* (Nath *et al.*, *loc. cit.*) with special reference to its beneficial influence in relieving the diabetic symptoms of various kinds by way of restoring proper metabolism in the system.

That amellin can be of great help in oxidizing keto bodies in the animal system has been proved conclusively by the observations *in vivo* (Table VII and Graph 4). Injection of 200 mg. of amellin simultaneously with 300 mg. of acetoacetic ester, in the normal rabbits, has checked accumulation of large concentration of keto bodies in the blood and has also caused their disappearance from the blood to a far greater extent. Graph 3 shows how the diabetic blood is deficient in some such factor which is essential for ketolysis and hence possibly of bringing about metabolism of other constituents such as lipoids and carbohydrates as postulated by Nath in 1941-42 and indicated by Nath and Chowdhury (1943a).

SUMMARY.

1. Normal plasma has been found to cause great influence in the process of oxidation of acetoacetic acid with hydrogen peroxide in alkaline medium.

2. Oxidation of acetoacetic acid takes place *in vitro* with normal plasma alone in alkaline medium even without hydrogen peroxide.
3. Amellin, the new anti-diabetic plant product, also causes enormous effect in the process of ketolysis *in vitro* with H_2O_2 in presence of alkali.
4. The nature of oxidation caused by glucose + H_2O_2 differs greatly with that with either amellin + H_2O_2 or plasma with or without H_2O_2 . There is observed an initial latent period in case of the former while in the latter cases the acceleration begins at the very beginning.
5. Plasma from the diabetic blood is far less effective in helping the process of ketolysis *in vitro*, thus indicating deficiency of some factor in it.
6. Amellin has been found to cause ketolysis *in vivo* while injected simultaneously with acetoacetic ester in normal rabbits.

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In Memoriam

MOHANDAS KARAMCHAND GANDHI, the Architect and Father of the Indian Nation and one of the greatest spiritual leaders in the history of the world, was born on the 2nd October, 1869, at Porbandar in Kathiawar. He died on the 30th January, 1948, at New Delhi.

By the tragic death of Mahatma Gandhi the world has lost one of its greatest physicians, a physician who saw beyond the ills of the body to the far greater ills of the soul, and with divine wisdom sought to heal the soul of the world in torment.

A man of great vision and determination, an exceptionally gifted psychologist, who knew the spirit, pulse and character of his countrymen like no other man has ever known, he fought against poverty and disease with weapons peculiarly his own, self-sacrifice, penance and fasting, weapons which he knew would inspire others to a greater nobility of character.

Mahatmaji was one of the greatest humanitarians and social reformers of the world, who all his life laboured for improving the standards of sanitation and education of his people, and for the eradication of slums and untouchability in his country.

As a young man Mahatma Gandhi, then fighting India's cause in South Africa, laid aside his political activities during the Boer War for the sick and wounded. He organized and led an Ambulance Unit for the British, recruiting Indians in South Africa as his stretcher-bearers. This Unit did 'heroic work at the front, very often under fire, and with

Gandhi always in the fore'. Again, during the Zulu Rebellion he organized another similar Unit for the British, for work amongst the Zulu wounded prisoners of war. This Unit received 'the highest praise for its work'. In 1914 when the war broke out in Europe, Gandhiji asked for and was allowed to raise an Indian Ambulance Unit in England, from Indians in Great Britain. Unfortunately, he was forced to give up this work because of serious illness and had to return to India.

To Mahatma Gandhi, dirt and disease were synonymous terms. His keen interest in sanitation brought many a rebuke to those lacking sense of hygiene and cleanliness. He always strove by practice and precept to teach the importance of cleanliness—cleanliness in environment, in body and in mind. His simple healthy diet kept his seemingly frail body in a very fit condition and, but for the cruel hand of an assassin, who can say how long, perhaps how very much longer, he would have been with us to guide us in his matchless way.

Dr. Stanley Jones, writing in 1939 in his book entitled 'Along the Indian Road', states 'The colour of the blood which Mahatma Gandhi has written into the history of India will be living and vivid thousands of years from now.....We shall all step up on the frustrate body of the little man and when we pass on and he arises from the dust we shall then see how truly tall and great he was. We are now too close to him to see his greatness.'

Mahatma Gandhi's supreme self-sacrifice and the laying down of his life to cleanse us of our sins and wickedness should not be in vain. We must do our best to follow his teachings for which he lived and died.

Kasauli,
23rd February, 1948.

M. L. A.
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SALMONELLA POONA ISOLATED FROM A GUINEA-PIG.

BY

CAPTAIN J. F. FREEMAN.

(From the Central Military Pathological Laboratory, India, Poona.)

[Received for publication, November 20, 1947.]

INFECTION by members of the salmonella group occur in men, animals and birds giving rise either to a fever, septicæmia or gastro-enteritis.

In man every endeavour is made to isolate the infecting organism either by blood culture or by culture of the fæces and urine. The use of Leifson's desoxy-cholate-citrate-agar as modified by Hynes (1942) or D.E.C. medium (Panja and Ghosh, 1943) are the two selective media admirably suited for the isolation of organisms of the salmonella group. Whenever the organism is isolated, its identity is fully established.

In animals and birds no such thorough investigations are usually made. However, whenever the cause of infection is investigated, the usual salmonella one isolates is either *Bacterium typhimurium* or *Bacterium enteritidis*. Both these organisms are a source of annoyance to laboratory workers, not only because they contribute to epidemics which occur from time to time in the animal house, but also to the fact that these organisms lie dormant in the animals and only make themselves evident when the animals' resistance is lowered, for example, by animal passage of a virus, thus vitiating any experiment one is undertaking.

In a laboratory in the India Command an epizootic infection among the guinea-pigs occurred depleting nearly half the stock of these animals. The main symptom these animals suffered from was that of a severe gastro-enteritis. Nothing of significance was observed on post-mortem examination.

Culture from the heart blood from one of the three animals that died on one single day revealed the presence of an organism of the salmonella group which was sent to this Laboratory for further identification.

The animals were fed on dry fodder obtained from military sources. Substitution of dry fodder by green vegetables resulted in a decrease in the mortality and finally in the arrest of the disease.

BIOCHEMISTRY.

The biochemical findings indicated that the organism isolated was none other than a member of the salmonella group.

SEROLOGY.

Identification of the somatic antigen.—A 'O' suspension of the organism, which in this paper will be designated as X, was prepared and tested on the slide for evidence of agglutination. The following are the results as stated against each of the 'O' sera used :—

Bacterium paratyphosum A 'O'.—

(I), II, XII. Representing group A	No agglutination.
------------------------------------	-----	-----	-------------------

Bacterium paratyphosum B 'O'.—

(I), IV, (V), XII. Representing group B	" "
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Bacterium paratyphosum C 'O'.—

VI, VII. Representing group C	" "
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Bacterium typhosum.—

XI, XII. Representing group D	" "
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Bacterium anatum.—

III, X, XXVI. Representing group E	" "
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Bacterium poona.—

XIII, XXII. Representing other groups	Agglutination.
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Agglutination absorption tests.—

<i>Bact. poona 'O' (1,000) versus X 'O'</i>	= 1,000
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<i>Bact. poona (250) — X 'O' versus Bact. poona 'O'</i>	= Nil.
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<i>Bact. poona (250) — X 'O' versus X 'O'</i>	= Nil.
---	-----	-----	--------

Identification of the flagellar antigens.—

Phase I :

<i>Bact. poona 'H' (1,000), phase I versus X 'H', phase I</i>	= 1,000
---	-----	-----	---------

<i>Bact. poona 'H' (250), phase I — X 'H', phase I versus</i>			
---	--	--	--

<i>Bact. poona 'H', phase I</i>	= Nil.
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<i>Bact. poona 'H' (250), phase I — X 'H', phase I versus</i>			
---	--	--	--

<i>X 'H', phase I</i>	= Nil.
-----------------------	-----	-----	--------

Phase II :

<i>Bact. poona 'H' (1,000), phase II versus X 'H', phase II</i>	= 1,000
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<i>Bact. poona 'H' (250), phase II — X 'H', phase II</i>			
--	--	--	--

<i>versus Bact. poona, phase II</i>	= Nil.
-------------------------------------	-----	-----	--------

<i>Bact. poona 'H' (250), phase II — X 'H', phase II</i>			
--	--	--	--

<i>versus X 'H', phase II</i>	= Nil.
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The somatic agglutination absorption test indicates that the 'O' antigenic structure may be similar to that of *Bact. poona*. It is presumed from the flagellar agglutination absorption tests that the organism under investigation may be *Bact. poona*. That this presumption is correct can only be confirmed by the 'mirror test'.

Reciprocal agglutination test.—

Somatic antigen :

X 'O' (1,000) versus <i>Bact. poona</i> 'O'	= 1,000
X 'O' (250) — <i>Bact. poona</i> 'O' versus X 'O'	= Nil.
X 'O' (250) — <i>Bact. poona</i> 'O' versus <i>Bact. poona</i> 'O'	= Nil.

Flagellar antigens :

X 'H', phase I (10,000) versus <i>Bact. poona</i> 'H', phase I	= 10,000
X 'H', phase I (250) — <i>Bact. poona</i> 'H', phase I versus X 'H', phase I	= Nil.
X 'H', phase I (250) — <i>Bact. poona</i> 'H', phase I versus <i>Bact. poona</i> 'H', phase I	= Nil.
X 'H', phase II (1,000) versus <i>Bact. poona</i> 'H', phase II	= 1,000
X 'H', phase II (250) — <i>Bact. poona</i> 'H', phase II versus X 'H', phase II	= Nil.
X 'H', phase II (250) — <i>Bact. poona</i> 'H', phase II versus <i>Bact. poona</i> 'H', phase II	= Nil.

The reciprocal agglutination absorption tests are self-explanatory. The agglutinins present in the organism under investigation are completely absorbed both by the somatic antigen present in *Bact. poona* 'O' as well as by the flagellar antigens, phases I and II. Neither the somatic antigen nor its homologous agglutinin contained the factor I.

It is conclusively proved that the organism under investigation is *Bacterium poona*. According to the Kauffmann-White diagnostic scheme, the organism has the antigenic formula XII, XXII, Z \leftrightarrow 1, 6

SUMMARY.

A short description of an organism isolated from a guinea-pig having the biochemistry and antigenic structure of salmonella poona is given.

Bact. poona was originally isolated from the faeces of a child suffering from acute gastro-enteritis and its antigenic structure determined by Bridges and Scott (1935).

Since then, the organism has been isolated from time to time, in cases of gastro-enteritis in man. It is, however, not known whether *Bact. poona* has previously been isolated from animals.

This is the second occasion that *Bact. poona* has been recorded in India.

The author's thanks are due to the D. M. S. in India and Lieut.-Colonel F. J. Hallinan, R.A.M.C., Officer Commanding, Central Military Pathological Laboratory, Poona, for permission to publish this paper.

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VARIATION IN THE VIRULENCE OF *M. TUBERCULOSIS* AND ITS CORRELATION WITH THE CLINICAL TYPE OF TUBERCULAR DISEASE.*

BY

R. G. DHAYAGUDE, M.D.,

AND

B. R. SHAH, M.D.

(From the Department of Pathology & Bacteriology, Seth G. S. Medical
College, Parel, Bombay, India.)

[Received for publication, November 17, 1947.]

IN the production of tuberculosis, which is a major problem in India, there is an interplay between the two factors, viz. the *Mycobacterium tuberculosis* and the host in which it produces the disease. In so far as the host is concerned it is well known that age, sex, occupation, overcrowding and unhygienic mode of living wield an undoubted influence in the ætiology of the disease. Due to the industrialization and urbanization in the war period features such as overcrowding, environmental lack of hygiene and undernutrition have been realized, more than before, as very important causes which need attention from the public health point of view if the spread of tuberculosis is to be controlled.

In respect of *Mycobacterium tuberculosis* a large amount of work on its bacteriology has been carried out since the time Koch first discovered it as the causative organism of the disease. We know quite a good deal about morphological and cultural characters of the various types of *M. tuberculosis*. In so far as the biological characters are concerned, in one important respect our knowledge is, however, still deficient. Topley and Wilson in their book on 'Principles of Bacteriology and Immunity' on page 423 state 'that very little exact information however based on an adequate number of animal tests is available about the

* This investigation was carried out under the auspices of the Indian Research Fund Association, the junior author having been awarded a Research Fellowship.

difference in virulence of freshly-isolated strains of the same type or about factors which are responsible for changes in virulence occurring *in vitro* and *in vivo*. Since virulence is bound to play an important rôle in determining infection it was decided to carry out experiments on the variation in the virulence of the *M. tuberculosis* of the hominis type.

Virulence has been defined as the power of a micro-organism to invade tissues and produce pathogenic effects on the infected animal. That virulence of different strains of micro-organisms varies and is an important factor to be reckoned with in experimental infections is well known. Lockhart (1926) experimented with two strains of *Salmonella typhimurium* 'A 52' and 'Ellinger' strains and showed conclusively that these two strains showed a marked variation in their virulence. That certain epidemics of bacterial diseases show a marked virulence probably due to the heightened virulence of the causative organism is a conception which is accepted on all hands. In the case of *M. tuberculosis*, however, we are not as yet sure whether there does or does not exist any variation in its virulence.

Grown in artificial media *M. tuberculosis* under experimental conditions can be made to lose its virulence has been shown by the production of BCG and RI strains of Trudeau which lost their virulence to such an extent that large doses of either of the two organisms can be injected without producing infection in experimental animals. What we are concerned with in this piece of work is the virulence of freshly-isolated strains of *M. tuberculosis*. Griffith (1924) who worked on freshly-isolated strains from skin lesions showed that on injection into susceptible animals they produced only very mild lesions. He concluded therefrom that skin tuberculosis is produced by a less virulent strain of *M. tuberculosis*. Griffith is not the only worker who believed in this variation in the virulence of *M. tuberculosis*. Workers such as Boquet and Saenz (1940) have even gone to the length of dividing freshly-isolated strains in three types: those with normal virulence, high virulence and attenuated virulence. As opposed to this the general consensus of opinion is that 97 per cent of strains of freshly-isolated *M. tuberculosis* are normal in their virulence. In view of this opinion and the conclusion drawn by Topley and Wilson, it was considered worth while to carry out adequate number of animal experiments and see whether any conclusions could be arrived at on this important problem.

Tuberculosis runs a varied type of course in human beings. In a large majority of cases it runs a chronic course but in others it takes a rapidly advancing turn and it was thought that while the question of variation of virulence was being investigated it would be interesting to incorporate observations regarding the correlation between the germs found in early, rapidly-advancing and chronic types of human cases to its virulence as observed in susceptible animals.

STRAINS OF *M. tuberculosis*.

— Strains of tubercle bacilli which were utilized for the purpose of these experiments were isolated from a variety of material such as sputum, bone lesions, caseous lymph nodes, cerebro-spinal fluid, etc. In all 76 strains were isolated but it became possible to utilize only 24 strains for animal experiments. Table I gives the type of material from which these 24 strains were isolated. It will be noticed that 18

strains were derived from the sputum, i.e. pulmonary in origin, and the remaining 6 of non-pulmonary sources.

TABLE I.

(i) Sputa	18	Pulmonary.
(ii) Pus (from bone abscess, etc.)			2	Non-pulmonary.
(iii) Puncture fluids (pleural, pericardial, peritoneal)				...	2	
(iv) Lymph glands	1	
(v) Cerebro-spinal fluid		1	
TOTAL					24	

Method of isolation.—Sputa, pus and contaminated fluids were treated with 15 per cent sulphuric acid for 15 minutes and all traces of acid were removed by washing the sediment twice with sterile distilled water. This not only concentrated the material but rendered it suitable for direct culture as all contaminating organisms were destroyed by the acid. For primary isolation the cultural method was utilized for two reasons :—

- (i) In order to avoid any possible change in virulence of the organism during its passage through the animal.
- (ii) It had been shown by previous experience of Dhurandhar (1941) that cultural method was superior to animal inoculation for primary isolation of tubercle bacilli. The medium used was a modified Lowenstein's medium having the following composition :—

Mono-sodium dihydrogen phosphate	...	1 g.
Sodium citrate	...	1 g.
Magnesium sulphate	...	1 g.
Asparagin	...	3 g.
Distilled water	...	1,000 c.c.

To 150 c.c. of this solution were added 12 c.c. of glycerine, 6 g. of potato starch, 5 eggs and 5 c.c. of 2 per cent aqueous solution of malachite green.

The sediment was planted on this medium and the tubes were corked and sealed with paraffin. They were then incubated at 37°C. Generally the growth became visible in about 10 days but sometimes it was delayed up to 25 days or so.

For experiments on animals as far as possible the primary culture was employed but in some instances the first subculture was used. It is well known

that on repeated subcultures, even under most suitable conditions of growth, organisms may tend to lose virulence as was demonstrated in case of RI strain of Trudeau.

Typing of the strain.—On isolation, the strain was typed to determine whether it belonged to the human or bovine variety by noting the cultural characters and the results of rabbit inoculation. All the strains isolated were of human type. Reports from other parts of this country (Ukil, 1942; Mallick, Aggarwal and Dua, 1942) also are in conformity with this finding. This is also borne out by the fact that tuberculosis is rare in Indian milch cattle and that Indian cows are more resistant even to experimental inoculation as compared to English breed (Liston and Soparkar, 1917).

Dose.—In order to produce infection in guinea-pigs different investigators have used different doses to suit their particular requirements. In our experiments we had to find a dose which will not be large and yet will invariably produce infection. We were guided in this by the work of Dhurandhar (*loc. cit.*) who showed by guinea-pig experiments that a number less than 1,000 organisms inoculated subcutaneously failed to give consistent results. He had found that a dose of 0.1 mg. produced infection in almost every animal so inoculated. It was on these considerations that a dose of 0.1 mg. of dry culture was selected and kept constant throughout. This quantity of the culture was suspended with the help of a drop of bile in 1 c.c. saline and was injected subcutaneously in the right groin of the guinea-pig.

Animals.—Our stock of guinea-pigs was a mixture of those obtained from the Haffkine Institute, Bombay, the Central Research Institute, Kasauli, and from Baroda. All the healthy animals were kept separate for breeding and the young ones used in this experiment were bred from this stock. As soon as a female became pregnant, it was separated from others. The young ones from a female were kept with the mother till they were one month old and then they were separated and numbered. Only pairs of the same litter were selected for use in these experiments when they gained a weight between 350 g. and 450 g.

In one of the animals of a pair of the same litter was injected one strain of *M. tuberculosis*, while in the other another strain was injected. To inoculate each strain about 12 animals were used, the mates of which were injected with another strain to gain a comparative idea of the virulence of the two strains to be compared. The object of using paired animals was to equalize the hereditary factors of susceptibility or resistance as far as possible.

One c.c. of the bacterial suspension prepared as mentioned above was injected subcutaneously in the right groin using a Luer-lock syringe. As far as possible, all animals were injected at the same time with the same strain and an equal number of animals with the strain to be compared.

It must be stated here that although originally intended it was not possible to use all the 12 animals with each of the strains. The difficulty of procuring sufficient number of animals and getting them to breed to produce adequate number of mates of the same litter came in the way of keeping the uniformity. However, with none of the strains were the number of animals used less than six, while in the case of some strains as many as 23 animals were used. As will be seen from Table II

as far as possible the same number of animals were used in each of two strains injected at a time. The total number of animals utilized in these experiments were 267 in all:—

TABLE II.

Serial number of strain.	Laboratory number of strain.	Total number of animals (guinea-pigs).	Serial number of strain.	Laboratory number of strain.	Total number of animals (guinea-pigs).
1	36	12	14	64	12
2	38	12	15	82	14
3	24	6	16	110	14
4	Q/13474	15	17	105	8
5	219	10	18	20	7
6	S/1526	10	19	13	6
7	231	12	20	R/767	12
8	232	12	21	93	15
9	Q/97941	23	22	108	15
10	98	8	23	127	9
11	313	12	24	133	9
12	12	6	Total number of guinea-pigs used ...		267
13	Q/11913	8			

Experience of last 20 years in this Laboratory has shown that there is no incidence of spontaneous tuberculosis in guinea-pigs from our stock. The animals were kept under identical conditions of housing and food. Each of the injected animals was kept in a separate cage.

It was thought that climate, as is present in Bombay, may have some effect on the resistance of the guinea-pigs. Two of our strains were, therefore, sent to Professor Gharpure, who was then stationed at Quetta which was very cool

at that time. His findings were identical with those of ours (Gharpure, 1947—personal communication).

Experimental infection.—Progress of the disease was noted by keeping a weekly record of weight and temperature and observation of evidence of enlarged lymph glands, etc. It is surprising to note that compared with the marked emaciation in human disease majority of guinea-pigs which on post-mortem examination showed an extensive disease did not show a loss of weight. Either the weight was constant or sometimes even an increase in the weight was noticed. Only in a very few animals was loss of weight evident. This is in conformity with the findings of Dhurandhar (*loc. cit.*). The same can be said about temperature. We also could corroborate his observation that there was no marked rise in temperature in these infected animals. These two well-known criteria of infection in a guinea-pig were not of much use in judging the clinical course of the disease in these animals.

It is generally said that the guinea-pig shows evidence of infection in a period of six weeks. An observation period of 14 weeks was fixed up to be on the safe side. If after inoculation the animal died during this period, a post-mortem examination was carried out and the extent of the disease was observed. At the end of this period all the surviving animals were autopsied and the progress of the infection recorded.

If the two animals derived from the same litter and injected with two different strains keeping the dose and time of observation, the nutrition and housing of the animals constant, showed a difference in the extent of the disease, the conclusion drawn was that the virulence of these two strains varied. As about 12 animals were inoculated with each of the strains, the factor of individual animal variation, in regard to its reaction to *M. tuberculosis* was also eliminated.

The extent of the disease was recorded in the protocols by signs + to +++++. An error due to personal equation in judgment may here crop in so that a lesion which can come under ++ may be either called + or +++++. This error, however, becomes less and less as experience accumulates. Table III gives the connotation of these signs :—

TABLE III.

+	•	A moderate or marked enlargement of right superficial inguinal and right deep inguinal glands with or without formation of a local abscess at the site of inoculation.
++		Involvement of superficial inguinal glands of the left side together with above and a. enlargement with or without caseation of common iliac and para-aortic glands.
+++		All the glands mentioned above <i>plus</i> tubercles in the spleen and liver.
++++		Generalized tuberculosis involving all the viscera.

Although in many cases the lesions were evident to naked eye, not to miss earliest evidence of infection, histological examination of the tissues taken out at autopsy was invariably carried out and the extent of the disease was recorded.

The nature of the virulence of a strain was determined by the average result of infection obtained in the number of animals injected. The following two illustrations would give an idea as to how the estimate was arrived (see Tables IV and V):—

TABLE IV.

Strain No. 9 (Q/97941) isolated from ileo-psoas abscess pus in a female patient aged 32 years, injected on 18th May, 1944.

Serial number of guinea-pig.	Laboratory number of guinea-pig.	WEIGHT IN G.		TEMPERATURE, °F.		Died or killed.	Extent of lesions.
		Beginning.	End.	Beginning.	End.		
1	546	375	560	100.6	101.6	K	++
2	549	300	400	98.0	99.8	K	+++±
3	552	360	510	100.6	100.2	K	+++±
4	554	380	400	99.7	100.0	K	+++
5	556	310	560	98.8	102.0	K	++±
6	557	310	400	100.0	101.4	K	++
7	562	430	450	99.6	100.8	K	+++
8	565	305	460	100.7	101.6	K	++±
9	567	300	370	98.8	100.2	D	±
10	558	490	540	101.2	101.2	K	++++
11	570	360	490	100.2	100.4	K	+++±

K = Killed at the end of 14 weeks.

D = Died before 14 weeks.

+ indicates the extent of the lesion (see text).

± indicates intermediate grade of extent of the lesion.

Variation in the Virulence of *M. tuberculosis*.

TABLE V.

Strain No. 20 (R/767) isolated from cerebro-spinal fluid of a male child aged 5 years, injected on 18th May, 1944.

Serial number of guinea-pig.	Laboratory number of guinea-pig.	WEIGHT IN G.		TEMPERATURE, °F.		Died or killed.	Extent of lesions.
		Beginning.	End.	Beginning.	End.		
1	545	330	450	100.2	100.2	K	+
2	547	390	550	100.0	100.2	K	+
3	548	345	310	99.6	102.3	D	+±
4	551	410	510	101.2	100.0	K	+
5	553	385	560	100.1	100.0	K	+
6	555	335	430	99.4	99.2	K	+
7	561	365	510	100.0	101.8	K	+±
8	563	325	510	99.2	102.0	K	+±
9	566	300	400	99.7	100.0	K	++
10	568	300	400	99.0	102.2	K	+
11	569	300	550	100.6	100.2	K	+±
12	571	300	560	99.0	102.0	K	++

K = Killed at the end of 14 weeks.

D = Died before 14 weeks.

+ indicates the extent of the lesion (*see text*).

± indicates intermediate grade of extent of the lesion.

It will be observed from Tables IV and V that the results obtained were usually clear cut and there was not much difficulty in classifying the strain in a particular category.

It will be noticed that in order to make the observation reliable, every effort was made to carry out the experiments under identical conditions and the only variant which was kept were the two strains of organisms used. Having taken all the precautions detailed above it would not be wrong to conclude that freshly-isolated strains of *M. tuberculosis* do vary in their virulence as has been observed in the case of many other bacteria.

According to these observations 24 strains utilized for the purpose could be divided into three groups depending upon the extent of the lesions they produced. Out of the strains, 13 were of high virulence, 6 of moderate virulence and 5 of low virulence. There was no strain which could be called avirulent by the standard applied in these experiments.

CORRELATION WITH CLINICAL TYPE TUBERCULAR DISEASE.

In order to correlate the virulence of the strain with the clinical conditions of the patient from whom it was isolated, the cases were grouped from a clinical point of view into three classes:—

- (i) Early cases.
- (ii) Advancing tuberculosis.
- (iii) Chronic fibroid phthisis.

This grouping was made after a careful consideration of the clinical history, duration of the disease, weight, temperature and pulse records together with *x*-ray pictures, sputum examination and a follow-up of these cases as far as was possible.

Cases which were classified as early cases were those in which an early lesion was detected in the lung, which on treatment in sanatorium with artificial pneumo-

CHART.

GRADE OF VIRULENCE	SOURCE OF THE STRAIN		TOTAL NUMBER OF STRAINS
EARLY CASES	PULMONARY	NON-PULMONARY	
+			
++	●●	● (PERICARDIAL FLUID)	3
+++	●●		2
++++	●	● (PLEURAL FLUID) ● (ABSCESS PUS)	3
TOTAL	5	3	8
ADVANCED CASES			
+	●●●		3
++	●●	● (C.S.-FLUID)	3
+++	●●●		3
++++	●●		2
TOTAL	10	1	11
CHRONIC CASES			
+	●	● (MESENTERIC GLANDS)	2
++			
+++			
++++	●●	● (ILIO-PSOAS ABSCESS)	3
TOTAL	3	2	5
TOTAL NO. OF STRAINS	18	6	24

thorax showed rapid improvement. Those in group (ii) are cases which showed rapidly-advancing disease and which steadily went downhill and ultimately succumbed to the disease. The third group speaks for itself.

The Chart on page 87 shows the clinical status, the grade of virulence of *M. tuberculosis* isolated from the case and their division into pulmonary and non-pulmonary strains. It will be seen that the same type of variation in the virulence is observed whether the strains are pulmonary or non-pulmonary in origin. As illustrated in Tables IV and V, a strain which was isolated from the cerebro-spinal fluid in a case of tubercular meningitis showed ++ virulence as compared with a strain which was isolated from a psoas abscess. The latter showed a ++++ virulence as indicated in the Chart. These two instances also bring out the fact that the virulence is not correlated with the clinical disease in the patient. Tubercular meningitis is a graver disease characterized by rapid progress than psoas abscess which may run a mild chronic course for months and sometimes years. The same observations apply to the strains of pulmonary origin.

The 24 strains (pulmonary and non-pulmonary) submitted to animal experiments were derived as follows (Table VI):—

TABLE VI.

				Strains.
Early cases	8
Advanced tuberculosis		11
Chronic tuberculosis		5
TOTAL	24

Table VII shows the extent of the lesions produced by these strains:—

TABLE VII.

Clinical nature of the case from which the strain was derived.	NUMBER OF STRAINS.			EXTENT OF LESIONS.			
	Pulmonary.	Non-pulmonary.	Total.	++++	+++	++	+
(i) Early cases ...	5	3	8	3	2	3	...
(ii) Advanced tuberculosis.	10	1	11	2	3	3	3
(iii) Chronic phthisis ...	3	2	5	3	2

It will be seen from Table VII that the virulence of the strain has no relation with the clinical type of case from which it is isolated. Three out of a total of five strains isolated from cases of chronic fibroid phthisis were of high virulence, while strains of all grades of virulence were isolated from cases of rapidly-advancing tuberculosis.

SUMMARY AND CONCLUSIONS.

1. All the 24 strains of *M. tuberculosis* including 6 from non-pulmonary sources belonged to the human variety. No strain of bovine type was isolated, indicating its rarity in India.

2. A study of 24 strains of tubercle bacilli isolated from different sources is made with a view to measure the virulence of each strain and compare it with others by means of animal experiments.

3. The process of isolation indicating the source and the technique of the experiment is described in detail.

4. Two conclusions have been arrived at as a consequence, viz.:—

(a) Freshly-isolated strains of tubercle bacilli vary in virulence.

(b) This virulence cannot be correlated to the clinical type of case from which the strain is isolated.

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ANTISEPTICS OF THE ACRIDINE SERIES.

Part II.

EFFECT OF CHANGING CHLORINE ATOM IN N-SUBSTITUTED 3-METHOXY-9-AMINO-ACRIDINE FROM POSITION 5 TO 7 AND 8.

BY

SURJIT SINGH, M.Sc. (Hons.),

AND

J. R. CHAUDHRI, L.R.C.P. (Lond.), M.R.C.S. (Eng.), D.P.H. (Cantab.).

(From the East Punjab University, Simla.)

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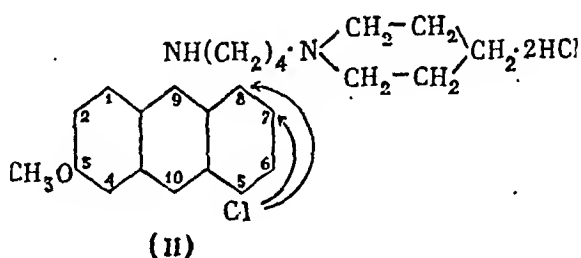
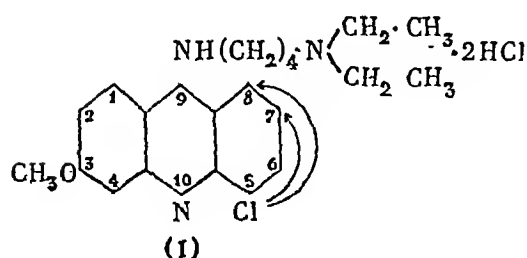
THIS paper is in continuation of the work being done to find out if there is any relation between chemical constitution and antiseptic activity amongst the acridines. In this series we have fixed the position of the methoxy group at 3 and basic side chain at 9 and then shifted the chlorine from position 5 to 7 and 8. The effect of chlorine at position 6 could not be studied as these compounds could not be obtained in a pure state.

Two identical compounds of each series were then submitted for bacteriological experiments: In one case diethyl compounds (I) were used and in the other piperidine compounds (II). There is a small difference in the molecular weight (mol.-wt. of I—458.5 and of II—470.5), i.e. one carbon atom more, but II was a closed-chain compound instead of an open-chain one. The difference in the activity (as reported in the results) is, however, considerable. The testing technique was essentially the same as described in Part I of this series (Surjit Singh and Chaudhri, 1947), the bactericidal values being determined by incubating for 48 hours at 37°C. the mixtures of various concentrations of compounds under test and the test

organism, using agar, MacConkey and glucose broth as the nutrient media. Absence of growth in all the three media was taken as evidence of bactericidal action.

The following derivatives were used in the experiments:—

- (1) 3-methoxy-5-chlor-9-(δ -diethyl-amino-butyl)-amino-acridine 2HCl.
- (2) 3-methoxy-7-chlor-9-(δ -diethyl-amino-butyl)-amino-acridine 2HCl.
- (3) 3-methoxy-8-chlor-9-(δ -diethyl-amino-butyl)-amino-acridine 2HCl.
- (4) 3-methoxy-5-chlor-9-(δ -piperidine-butyl)-amino-acridine 2HCl.
- (5) 3-methoxy-7-chlor-9-(δ -piperidine-butyl)-amino-acridine 2HCl.
- (6) 3-methoxy-8-chlor-9-(δ -piperidine-butyl)-amino-acridine 2HCl.



The results are summarized in Table I:—

TABLE I.

Bacteriostatic and bactericidal values in 0.1 per cent glucose broth of the compounds used in the experiments.

Compound number.	<i>Staph. aureus.</i>		<i>Strept. haemolyticus.</i>		<i>Bact. coli.</i>		<i>Proteus vulgaris.</i>		<i>Pseudo. pyocyaneus.</i>	
	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.	Bs.	Bc.
1	8	4	16	16	16	16	*	*	*	*
2†	8	2	8	4	2	2	*	*	*	*
3†	4	*	2	*	2	*	2	*	*	*
4	16	8	32	16	32	16	2	*	*	*
5†	64	8	128	32	4	2	2	*	*	*
6†	4	*	*	*	4	*	*	*	*	*

Notes.—(i) Bs. denotes the bacteriostatic dilution of 1 : in thousands.

(ii) Bc. denotes the bactericidal concentration of the drug, 1 part in so many thousand parts.

* Less than 1 : 2,000.

(iii)† Compounds Nos. 2 and 5 were supplied by the courtesy of Mr. Surjit Singh, M.Sc., and 3 and 6 by Mr. Apar Singh, M.Sc.,

Sachdeva (1943) has used mepacrine hydrochloride solution for the treatment of local sores. The compounds under examination are very closely related to it and should be non-irritant. This has been the case as seen from results given in Table II. The method used for testing the irritant properties being the same as described in the earlier paper (Surjit Singh and Chaudhri, *loc. cit.*).

TABLE II.

Compound number.	REACTION (COLOUR OF THE CONJUNCTIVÆ) AFTER					Conc. used.	REMARKS.
	5 minutes.	10 minutes.	30 minutes.	24 hours.	Control.		
1	No redness	No redness	No redness	White	White	1 : 250	There was absolutely no sign of uneasiness or restlessness in the rabbits and the eye in general showed no untoward symptom.
2	"	"	"	"	"	"	
3	"	"	"	"	"	"	
4	"	"	"	"	"	"	
5	"	"	"	"	"	"	
6	"	"	"	"	"	"	

DISCUSSION.

1. (a) The diethyl compounds have yielded remarkable results. 5 chloro-compound is the most active one followed by 7 chloro, which in turn possesses higher activity than the corresponding 8 chloro-derivative.

(b) In the piperidine compounds, 7 chloro-derivative shows great abnormality. It has high bacteriostatic values for *Staph. aureus* and *Strept. hæmolyticus*, but has low values for *Bact. coli*. This abnormality, however, vanishes when we examine bactericidal values; they approach only to those of 5 chloro-derivative except against *Streptococcus* where 7 chloro-compound seems to be superior. The 8 chloro-compound has lower activity than 5 and 7 as anticipated.

(c) The higher activity of 5 chloro-compounds may be due to their higher basicity. The order of basicity has not been determined but 5 chloro-derivatives are definitely more basic than the corresponding 7 and 8 chloro-compounds.

2. It seems that closed-ring substituents, such as piperidine ($C_5H_{10}N$), are better than open-chain compounds like diethyl ($C_4H_{10}N$). It is interesting to note that the molecular weights of these compounds differ only by 1 carbon atom, whereas the activity increase of the closed compounds is surprisingly high (compare 1 and 4 and 2 and 5).

3. All these drugs are almost ineffective against *Pseudomonas pyocyaneus* and *Protus vulgaris*, while activity against other organisms is moderate.

4. These compounds were found to be non-irritant. No attempt, however, was made to test their activity in presence of serum as none of these derivatives had shown any exceptional activity.

We owe our sincerest thanks to Professor Mahan Singh for the encouragement and the keen interest taken by him during the work. One of us (S. S.) is indebted to Balwant Singh Trust, Gujranwala, for the award of a research scholarship.

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HÆMATOLOGICAL STUDIES IN NORMAL PREGNANT INDIAN WOMEN.

BY

L. S. GHOSH, R. B. LAL, S. MITRA, MUKTHA SEN,

AND

K. K. MATHEN, C. G. RAHA, U. RAY, C. GHOSH.

(From the All-India Institute of Hygiene & Public Health, Calcutta.)

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ANÆMIA figures predominantly in almost all the investigations into the causal factors of high maternal mortality which have been carried out in this country. Thus, Balfour (1927), McSwiney (1927), Wills and Mehta (1930), Mudaliar and Rao (1932), Neal Edwards (1940), Mitra (1931), and Napier and Majumdar (1938) bring out its importance as may be seen from Table I.

It is true that these figures exhibit a considerable variation, but that is to be expected, because, in the first place, it is a matter of personal judgment as to what deaths should be ascribed to anæmia as the primary cause, and in the second place the state in which the patients are admitted to hospital and the adequacy of the treatment given to them, differentially influence mortality rates from various causes. Besides, it is likely that anæmia as the cause of death may operate with different intensities in different parts of the country and amongst persons of different social status. It must also be remembered that these figures relate mostly to hospital cases, and it is generally known that in this country the number

TABLE I.

Percentage of maternal deaths due to anæmia.

Authority.	Year.	Number of patients.	Percentage of maternal deaths due to anæmia.	Place of work.
Balfour	1927	150	42	Bombay and Assam.
McSwiney	1927	43	35	Calcutta.
Wills and Mehta	1930	50	30	Bombay.
Mudaliar and Rao	1932	436	12	Madras-city.
Neal Edwards	1940	...	18	Collected data from 39 women's hospitals all over India.
Neal Edwards	1940	...	23	Calcutta.
Mitra	1931	...	48	Calcutta.
	1933	163	32	Calcutta.
	1937	561	37	Calcutta.
Napier and Majumdar	1938	58	17	Cachar.

of pregnant women seeking hospital accommodation is relatively small, and quite a large percentage of them come to hospital because they are weak and anæmic. Be that as it may, the contrast with corresponding figures in other countries is striking. Thus, in England and Wales only 0·05 per cent and in Scotland 0·08 per cent of maternal deaths are ascribed to anæmia. It is, therefore, necessary that maternal anæmia should receive special consideration in this country if any advance in the reduction of the present high rate of maternal mortality is to be effected.

A large number of studies on anæmia in pregnancy has been carried out in this country, but the picture is rather confused. To clear up the position it is necessary to standardize the technique of sampling as also the nature and the techniques of blood examinations. Besides, it is necessary to ascertain the blood picture of normal pregnant women and to determine the permissible range of variation.

While in their authoritative Memorandum on Anæmia of Pregnancy in India, Napier and Das Gupta (1942) have given standard methods of blood examination, there is no satisfactory study to establish the normal blood picture in healthy pregnant women in India. The only available data recorded in the Memorandum appear to have been based on observations of a small number of coolie women in

Assam and some in Calcutta and Coonoor. No details of clinical examination or period of gestation are given. Radhakrishna Rao's (1938) studies suffer from the additional disadvantage that they relate to Coonoor which is situated at an elevation of 6,000 feet above the sea-level.

In the present communication it is intended to describe the standard blood picture of clinically normal pregnant women in Calcutta, who could be contacted through clinics and hospitals. The examinations were carried out according to standardized techniques and included total red cells, hæmoglobin (by weight), cell volume, red cell diameter, reticulocytes, total white cells, differential leucocyte count, platelet count, sedimentation rate, icterus index, serum cholesterol, serum calcium and serum phosphorus. The range of variation in the normal group, and also in a larger group including individuals with such minor clinical abnormalities as do not appear to materially influence the blood picture, has been worked out for each item of examination. We have also tried to present the data separately for the various months of pregnancy.

SELECTION OF SUBJECTS.

The subjects of the study were drawn from the following local sources :—

1. The Maternity & Child Welfare Clinic of the All-India Institute of Hygiene & Public Health (outdoor).
2. Chittaranjan Seva Sadan (indoor and outdoor).
3. The Sishu Mangal Pratisthan (indoor and outdoor).
4. Maternity Clinic of the Calcutta Branch of Red Cross Society (outdoor).
5. Maternity Clinic of Clive Jute Mills Co. (outdoor).
6. Lady Dufferin Hospital (indoor).

In all, 1,165 pregnant women were examined during the course of three years commencing from April 1941. In some instances repeated examinations were made on the same subjects, the total number of examinations being 1,245. Besides, 79 examinations were made post-natal. It was intended to collect the following information for each subject (for details see Schedule in *Appendix II*).

1. Sociological data: Age, economic status, religion, province of origin, habitual diet.
2. Clinical: History of previous illnesses, parity, month of gestation, physical condition.
3. Laboratory tests:
 - (a) Hæmatological as mentioned above.
 - (b) Examinations for detection of albumin and sugar in the urine and helminthic ova in the fæces.
 - (c) Blood for the presence of parasites and Kahn test.
 - (d) Biochemical examinations: Estimation of serum calcium, serum phosphorus and serum cholesterol.

For various reasons it was not found possible to complete all the examinations in every subject.

The details are shown in Table II :—

TABLE II.

Number of cases according to the nature of examinations made.

Clinical and sociological.	LABORATORY TESTS.						
	Hæmato-logical.	Prisco-Jones.	Blood parasites.	Kahn.	Biochemical blood.	Urine.	Fæces.
1,165	1,165	130	1,165	1,165	687	1,165	899

Actually 'complete' examination (not necessarily including biochemical) was made for 899 pregnant women. The total number of complete examinations including 'repeats' was 977. In addition complete blood examination was made for 79 women, 6 to 8 weeks after delivery.

COLLECTION OF MATERIAL.

Blood.—Five c.c. of venous blood were collected in an Erlenmeyer flask, containing 0.05 c.c. of 20 per cent solution of potassium oxalate. The flask was agitated gently for 3 minutes to ensure thorough mixing. The blood so collected was used for the estimation of hæmoglobin, icterus index, cell volume, sedimentation rate, bilirubin, red cell count, white cell count and reticulocyte count. From the last drop slides were made for differential leucocyte count, blood platelet count and measurement of red cell diameter. At the same time 3 c.c. of blood were collected in a sterile bottle to provide serum for Kahn test. A further 15 c.c. of blood were drawn to obtain sufficient serum for the estimation of calcium, phosphorus and cholesterol.

Other materials were obtained in the usual way.

Laboratory methods.—For all hæmatological examinations standard techniques as described by Napier and Das Gupta (1942) were employed. The techniques employed in biochemical estimations are given in *Appendix III*. Standard apparatus conforming to the British Bureau of Standards (B.T.L.) were used. The chemicals used were of high purity and certified for use in biochemical investigations.

The subjects were divisible into a large number of groups. A description of the main groups is given below :—

Group I.—No clinical abnormality, no previous history of anæmia, Kahn test negative, no abnormality in urine, no evidence of helminthic infection and van den Bergh test negative. This group has been designated as the 'normal'. It comprised of 312 women with 359 completed blood examinations.

Group II.—Same as group I, except that ova of worms other than hookworm were detected in the fæces, includes 17 cases and 20 examinations.

Group III.—Same as group I, except that stools could not be examined, includes 131 cases with one repeat.

Group IV.—Same as group I, except that there was a trace of albumin in the urine, includes 141 cases and 10 repeats.

Group V.—Same as group IV, except that ova of worms other than hookworm were found in faeces, includes 15 cases, no repeats.

Group VI.—Same as group IV, but stools could not be examined, includes 64 cases with one repeat.

Group VII.—Same as group I, except that van den Bergh was positive, but did not exceed 0.4 units, includes 42 cases with two repeats.

Group VIII.—Same as group VII, but stool examination was lacking, includes 28 cases, no repeats.

Group IX.—Similar to group VII in all respects except that van den Bergh exceeded 0.4 units, includes 72 cases with 3 repeats.

Group X.—Same as group IX, but stool examination was lacking, includes 43 cases, no repeats.

Group XI.—Same as group IX, but, in addition, there was albumin in the urine, includes 75 cases, no repeats.

Group XII.—Same as group I, but hookworm ova were present, includes 31 cases, no repeats.

Group XIII.—Same as group XII, but, in addition, ova of other worms were present, includes 10 cases, no repeats.

Group XIV.—Hookworm ova present and van den Bergh test positive, but did not exceed 0.4 units, no other abnormality present, includes 11 cases with 5 repeats.

Group XV.—Kahn positive, van den Bergh test (direct or indirect) positive, but did not exceed 0.4 units, no other abnormality present, includes 26 cases with one repeat.

Group XVI.—Hookworm ova present and van den Bergh test (direct or indirect) positive, exceeding 0.4 units, no other abnormality present, includes 15 cases, no repeats.

Group XVII.—Evidence of kidney involvement, casts present in the urine, includes 25 cases with 2 repeats.

Group XVIII.—Includes all other groups showing clinical abnormalities, includes 107 cases with 5 repeats.

As may be seen from *Appendix IV*, the results of blood examination of groups II, III, IV, VII, IX and XII do not differ significantly from group I and may, therefore, be combined with it. This aggregated group will be referred to as the 'combined normal group', which included 746 women and 812 examinations. In discussing the results of examination we have taken into consideration group I (normal) and the 'combined normal' separately.

While theoretically the women included in an investigation, whose object is to describe the blood picture of normal pregnant women in India, should satisfy

statistical requirements of a representative sample, in practice this ideal cannot be fully attained. We must necessarily depend on subjects attending clinics and hospitals in a country where pregnancies are not notified and public agencies can contact the women only on voluntary basis. For purposes of comparison we are presenting a description of the social conditions of the population under consideration (see *Appendix I*). Within the limitations just stated, the selection of the women was made at random except in respect of the month of gestation with a view to include in the sample as even a distribution of various stages of pregnancy as possible. Special efforts were made to select women in earlier months of gestation. Thus, our approach in selecting the sample would appear to offer the best possible approximation to a theoretically sound sample in the circumstances, and we have reasons to believe that the results of investigation may be fairly appreciable to the bulk of Indian population residing in urban areas.

It would have been useful to know their pre-natal blood picture for purposes of comparison. Unfortunately, it is not possible to do so. However, we have presented a statement of blood picture of women of the combined normal group in the first trimester of pregnancy and of normal women as given by certain authors in this country and abroad in the hope that some idea of the normality of our subjects may be gauged thereby.

Our data are more directly comparable with those of Basu and Chatterji (1937). Their first series consists of unselected women living in Calcutta and the second series represents a selected sample of young Bengalee girls between 16 and 23 years of age living in South Calcutta. While these women are described as healthy, no detailed information about the nature of clinical examination is available and it is difficult to say whether the pregnant and the non-pregnant women represent identical social strata though it is not likely that there would be much divergence. It, therefore, would be hazardous to ascribe the differences noted below to the pregnant state.

Red cell count.—The number of red cells, according to Dieckmann and Wagner (1934), do not undergo material change during the first trimester and, therefore, they furnish a useful item for comparison. The red cell count in the present series does not differ significantly from that obtained by other Indian workers, except those recorded by Basu and Chatterji (*loc. cit.*) and by Dhar (1934) which are exceptionally low and to a certain extent that described by Napier and Das Gupta (1942). On the other hand the British and the American figures are decidedly higher but the value obtained by us is still within the normal range (*vide* Whitby and Britton, 1939). Thus, it would not be unfair to conclude that our subjects represented a cross section of the Indian community as judged from this important item of blood examination.

The mean corpuscular volume.—This is definitely lower than that recorded in other Indian and foreign studies with the exception of those by Napier and Billimoria (1937) amongst Assam coolies. The value obtained for the 'combined normal' group is approximately the minimum of the range for normal women, which according to Whitby and Britton (*loc. cit.*) is 78. In this connection, one may mention that there is a definite tendency for the red cells to swell up during the earlier months of pregnancy, but for some unknown reason, the cells of our subjects were of small size.

TABLE III.
Blood picture of combined normal group in the first trimester and of normal non-pregnant women in India and abroad.

Number.	Blood picture.	Present study (first trimester).	Sokhey (1938).	Napier and Das Gupta (1942).*	Sankaran and Rajagopal (1938).	Benjamin (1938).	Radhakrishna Rao (1938).
	Locality:—	Calcutta.	Bombay.	Calcutta.	Madras.	Delhi.	Coonoor.
	Number of cases:—	48	101	128	62	100	100
1	Erythrocyte count ...	4.41 ± 0.65	4.47 ± 0.33	4.015 ± 0.409	...	4.560 ± 0.250	...
2	Mean corpuscular volume ...	78.97 ± 7.43	85.53	86.82 ± 7.28	...	92.7	...
3	Mean cell diameter ...	0.49
4	Cell volume ...	34.38 ± 6.05
5	Volume index ...	1.00
6	Haemoglobin in g. per 100 c.c. ...	11.08 ± 1.95	12.99 ± 1.10	12.63 ± 1.01	13.73 ± 0.93	13.11 ± 0.81	15.81 ± 2.54
7	Colour index ...	0.90 ± 0.17
8	Mean corpuscular hemoglobin ...	25.40 ± 3.46	29.06	27.42 ± 2.89	...	28.76	...
9	Mean corpuscular hemoglobin concentration.	31.48 ± 2.93	32.86	31.57 ± 1.76	...	33.58	...
10	Saturation index ...	1.00
11	Reticulocyte ...	0.49 ± 0.33
12	Leucocyte count ...	8,200 ± 2,450	...	7,162 ± 1,765
13	Neutrophil count ...	5.875
14	Small lymphocyte count ...	1,320
15	Large lymphocyte count ...	477
16	Platelet count ...	221,000
17	Sedimentation rate ...	3.8

*Figures under this column are quoted from their Memorandum, referred to already.

The mean cell diameter is smaller in our sample than the value obtained by Basu and Chatterji and by Dhar so that the smaller size of the cell is not referable to thickness of the cell.

The cell volume of the 'combined normal' group is greater than that recorded by Basu and Chatterji. This is because the erythrocyte count in their case is exceptionally low.

The volume index in each case is unity or near about.

The hæmoglobin per 100 c.c. of blood is lower in the 'combined normal' group than the values recorded by other Indian workers, except by Napier and Billimoria (*loc. cit.*), and Napier and Majumdar (*loc. cit.*) in Assam. It is even lower than the value obtained by Basu and Chatterji, in spite of their smaller red cell count and the low cell volume. To a certain extent reduction in hæmoglobin is to be expected in pregnancy up to the end of second trimester, and since the colour index is unity, no serious notice may be taken of lower value of the hæmoglobin in the present series.

Other items of blood picture do not show abnormality. It may, therefore, be concluded that the 'combined normal' group represents normal healthy Indian women except as regards small size of red cells for which no explanation can be offered.

The distribution of the two groups under consideration according to months of gestation are given in Table IV:—

TABLE IV.

The distributions of clinical groups according to months of gestation.

MONTH OF GESTATION.								
	Under 4 months.	Under 5 months.	Under 6 months.	Under 7 months.	Under 8 months.	Under 9 months.	Over 9 months.	TOTAL.
Group I (normal) ...	48	42	48	47	49	50	75	359
'Combined normal' ...	103	73	91	126	136	116	167	812

The number of cases in each month of pregnancy is fairly large for the 'combined normal', but the distribution is not even and since the blood picture varies in pregnancy from month to month, it is not justifiable to strike an average. However, other workers' records are not available separately for each month of pregnancy, and the only way to carry out comparisons is to use averages for each item for the whole period of gestation (*vide* Table V).

TABLE V.

Blood picture of normal pregnant women in India and abroad.

	PRESENT STUDY.		Napier and Das Gupta (1937). Assam.	Bethel (1936) quoted by Napier. Michigan.	Napier and Billimoria (1937). Assam.	Napier and Das Gupta (1942). Calcutta.	Radhakrishna Rao (1938). Coonoor.
	Normal.	'Combined normal.'					
<i>Number of cases:—</i>	359	812	192	28	40	64	100
<i>Nature of cases:—</i>							
Blood picture.	Normal.	Noar normal.	Obvious anæmias excluded.	...	Coolies.
Erythrocyte count ...	4.17 ± 0.56	4.13	...	4.12	4.65 ± 0.62
Cell volume ...	32.78 ± 5.06	32.29
Mean red cell diameters ...	6.43 ± 0.53	6.49
Colour index ...	0.97 ± 0.17	0.96
Hæmoglobin in g. ...	10.51 ± 1.56	10.34	9.99 ± 1.72	11.85	10.70 ± 1.60	...	15.52 ± 2.2
Mean corpuscular volume ...	81.3 ± 7.87	80.97	...	92.00	72.10	86.83 ± 10.8	...
Mean corpuscular hæmoglobin ...	25.29 ± 4.4	25.11	23.80	26.62 ± 3.34	...
Mean corpuscular hæmoglobin concentration.	31.61 ± 2.50	31.64	32.60	30.57 ± 2.13	...
Saturation index ...	0.99	0.99
Volume index ...	1.01	1.00

TABLE V—*concl'd.*

Number of cases:—	PRESENT STUDY.		Napier and Das Gupta (1937). Assam.	Bethel (1936) quoted by Napier. Michigan.	Napier and Billimoria (1937). Assam.	Napier and Das Gupta (1942). Calcutta.	Radhakrishna Rao (1938). Coonoor.
	Normal.	'Combined normal.'					
Nature of cases:— Blood picture.	359	812	192	28	40	64	100
	Normal.	Near normal.	Obvious anemias excluded.	...	Coolies.
Reticulocyte count ...	0.63 ± 0.55	0.65
Icterus index ...	7.02 ± 2.27	6.99
Leucocyte count in thousands ...	8.48 ± 2.58	8.37
Neutrophil count in thousands ...	5.86 ± 2.57	6.59
Lymphocyte count (large) ...	4.79-95 ± 408.9	479.68
Lymphocyte count (small) ...	1,238.3	1,197.59
Monocyte count ...	148.28 ± 238.6	163.89
Eosinophil count ...	157.19 ± 206.8	161.45
Platelet count in thousands ...	200.0 ± 156.5	189.97
Sedimentation rate ...	4.50	4.75
REMARKS ...	Nationality Indian.	Nationality Indian.	Unpublished.	...

The erythrocyte count for our subjects compares favourably with that given by Bethel (1936) but it is somewhat smaller than the figure obtained by Napier and Billimoria (*loc. cit.*) for Assam coolies. Mean cell diameters are not recorded by other workers but the mean cell volume for our subjects is definitely smaller than that given by Bethel and also by Napier and Das Gupta (1942) for Calcutta but the figures for the Assam coolies is very much lower. It may, therefore, be concluded that smallness of the red cell is a characteristic of our women even during pregnancy, the cause of which needs further study. Unfortunately, comparative figures for other elements of blood for pregnant women are not readily available. Hæmoglobin in grammes per 100 c.c. is slightly lower for both the 'normal' and 'combined normal' as compared to the values given by Bethel. The value obtained by Radhakrishna Rao is exceptionally high but his investigations were conducted at a high altitude. The data given by other Indian authors correspond fairly closely to our figures.

A more complete picture of the blood of pregnant women of the present series is presented in Tables VI and VII in which separate data are given for each month of pregnancy after the first four months, separately for the 'normal' and the 'combined normal' groups. It will be observed that the red cell count decreases with the progress of pregnancy up to the seventh month followed by partial recovery at term and full recovery 8 weeks post partum. We have no knowledge of the red cell count of our subjects in non-pregnant state and since increase in plasma volume takes place in pregnancy fairly early (16 per cent in the 13th week in American women according to Dieckmann and Wagner, *loc. cit.*) data presented in Tables VI and VII do not convey full information about the decrease in red cell count with the progress of pregnancy compared with non-pregnant women. We have no knowledge of the amount of increase in plasma volume in Indian pregnant women but we know that practically all our subjects came under observation late in the third trimester when increase in blood volume had already taken place. If we assume the same amount of increase in plasma volume as was found by Dieckmann and Wagner in our women at the time they came under observation the number of red cells per c.mm. for non-pregnant women may be calculated. Thus, it will be seen that 22·7 per cent decrease in red cell count takes place by the 7th month of gestation. Dieckmann and Wagner present similar results.

Parallel changes are observed in the cell volume, but as compared with Dieckmann and Wagner's findings, the fall is greater (20·6 against 14 per cent). The mean corpuscular volume for the Indian women is definitely low (78·97 cu. microns) and their blood verges on microcytosis even in early pregnancy. Although a slight increase takes place at term it never approaches the high value of 94 as recorded by Dieckmann and Wagner. Post partum, the mean corpuscular volume lies just within the accepted normocyte range. The cell diameter decreases up to the 6th month.

The mean corpuscular hæmoglobin concentration diminishes slightly, the lowest figure being reached between 7 and 8 months. These alterations in the blood picture are to a certain extent the natural result of increase in the plasma volume which normally occurs in pregnancy. After a small rise it goes down again reaching the minimum value post partum because of the swelling of the cell. The mean hæmoglobin per cent suffers a loss early in pregnancy and continues to decrease

TABLE VI.
Blood findings for 'normal' women according to months of gestation.

Item of blood.	Months of gestation.						Average during gestation.	Post-natal (6 to 8 weeks).
	—4	—5	—6	—7	—8	—9	—10	
Red cell count ...	4.41	4.38	4.03	3.95	4.07	4.17	4.17	4.42
Cell volume ...	34.38	32.21	32.19	31.65	31.89	31.55	32.70	34.91
Hæmoglobin in g. per 100 c.c. ...	11.08	10.61	10.09	10.17	10.34	10.18	10.39	10.77
Mean corpuscular volume in cubic microns.	78.97	80.08	82.21	83.82	81.96	79.01	81.57	80.37
Mean cell diameter ...	6.49	6.39	6.30	6.50	6.54	6.44	6.48	...
Mean corpuscular hæmoglobin in micrograms.	25.40	24.97	25.08	26.15	24.75	24.83	25.34	24.22
Mean corpuscular hæmoglobin concentration in percentage.	31.98	31.56	31.14	31.48	31.23	31.93	31.86	30.44
Saturation index ...	1.00	1.02	0.97	1.00	1.01	1.00	0.97	0.96
Volume index ...	1.00	0.94	1.02	1.03	1.01	0.97	1.00	1.01
Colour index ...	1.04	1.05	1.07	1.03	1.04	1.04	1.05	1.00
Total leucocyte count ...	7,672	7,651	7,513	7,932	8,290	7,642	7,712	6,574
Neutrophil count per c.mm. ...	5,875	5,779	5,848	6,276	6,586	5,871	6,050	4,693
Sedimentation rate in mm. ...	3.8	4.0	4.7	4.4	4.9	4.8	4.8	3.15
Lymphocytes (large) ...	477	570	455	453	459	499	497	494.9
Lymphocytes (small) ...	1,320	1,302	1,210	1,203	1,245	1,272	1,165	1,386.1
Reticuleocyte count ...	0.49	0.45	0.75	0.53	0.60	0.69	0.69	0.44
Platelet count per c.mm. ...	221,000	202,000	167,000	170,000	156,000	205,000	180,000	202,500

TABLE VII.

Findings for combined normal groups.

Item of blood.	Months of gestation.							Average during gestation.
	—4	—5	—6	—7	—8	—9	—10	
Red cell count	4.41	4.28	4.04	3.89	4.05	4.16	4.13	4.14
Cell volume	34.71	32.05	31.62	31.01	31.31	31.65	32.63	32.14
Hæmoglobin in g. per 100 c.c.	10.96	10.58	10.09	9.87	10.20	10.28	10.45	10.35
Mean corpuscular volume in cubic microns	79.99	79.31	81.26	82.72	80.36	80.21	81.55	80.77
Mean cell diameter	6.52	6.57	6.35	6.50	6.52	6.44	6.51	6.49
Mean corpuscular thickness in μ	2.40	2.34	2.57	2.49	2.41	2.46	2.45	2.45
Mean corpuscular hæmoglobin in micromicrograms.	25.27	24.69	24.81	25.76	24.80	24.86	25.30	25.07
Mean corpuscular hæmoglobin concentration in percentage.	31.76	31.63	31.51	31.57	31.47	31.89	31.65	31.64
Saturation index	1.00	1.02	1.01	1.01	1.03	1.03	1.07	1.02
Volume index	1.00	0.98	0.99	1.01	0.98	0.97	1.00	0.99
Colour index	1.04	1.02	1.02	1.07	1.05	1.05	1.07	1.05
Neutrophil count per c.mm.	5,648	5,832	6,124	6,124	6,233	6,083	6,125	6,024
Sedimentation rate in mm.	3.9	4.2	4.7	4.8	5.2	5.1	5.0	4.7
Reticulocyte count	0.51	0.57	0.71	0.73	0.72	0.75	0.74	0.68
Platelet count per c.mm.	218,000	177,000	181,000	188,000	198,000	191,000	153,000	187,000

till the sixth month. The deficiency amounts to 21 per cent when it has reached its minimum value. Subsequent recovery is only partial. Seventeen per cent deficiency still remains, 8 weeks post partum. No significant or regular changes take place in volume, colour or saturation indices during gestation.

Generally speaking, the sedimentation rate is within normal limits but somewhat on the low side. There is slight increase in the 6th month after which no significant change takes place.

In the 'normal' group, leucocyte count is more or less constant and remains within normal limits, but there is a slight tendency to rise up to the eighth month followed by a fall which brings it back to the value of early months of pregnancy.

The differential count does not show any abnormality. The curve for the neutrophils runs parallel with the total count but the small lymphocytes suffer a slight decrease during gestation, lowest being recorded between the 6th and the 7th months. Blood platelets suffer a decrease in the mid-gestation period.

The post-natal data relate to all cases investigated during that period without eliminating the clinically unhealthy persons but their number is likely to be small as compared with normal women and discussion of these figures in connection with normal blood picture will not introduce significant discrepancies. It would appear that blood picture in the post-natal period shows a close approximation to that of the early period of pregnancy, except perhaps as regards the deficiency in the mean corpuscular hæmoglobin and mean corpuscular hæmoglobin concentration.

PRICE-JONES' CURVES.

When the blood picture of our 'normal' and 'combined normal' groups is compared with the general standards of Whitby and Britton (*loc. cit.*) for European women, and of Basu and Chatterji (*loc. cit.*) for Indian women or of Bethel (*loc. cit.*) for American pregnant women, it is found that the main difference lies in the smaller size of the red cells in our series. The reason why the mean corpuscular volume in the studies by Napier and Das Gupta for Calcutta women is relatively greater than that found in the present series is not clear.

While this information about the mean cell volume is of interest it gives no information about the degree of anisocytosis. Assuming normal relative thickness of the red cells this information can be best obtained by comparing the distribution of cell diameters with the standard Price-Jones' curves. Following the technique described by Napier, a series of unselected sample of 84 was taken from the normal group for measuring the cell diameters (500 cells for each individual). A similar investigation on 46 individuals was made from group III. Data have been separately analysed. For convenience we shall designate the two series as A and B in the discussion that follows.

Figs. 1 and 2 give the distribution of 42,000 (i.e. 84×500) cells of series A and of 23,000 (i.e. 46×500) cells of series B. Both these curves are skewed, the respective values of skewness being 0.075 and 0.013. Taking individuals separately it is found that in 25 instances in series A and in 11 instances in series B skewness is insignificant. Of the rest, 30 in series A and 14 in series B are significantly positive (mode to the left) and 29 and 21 respectively are significantly negative

FIG. 1.

Frequency distribution of red cell diameter of 84 women of series A each contributing 500 cells.

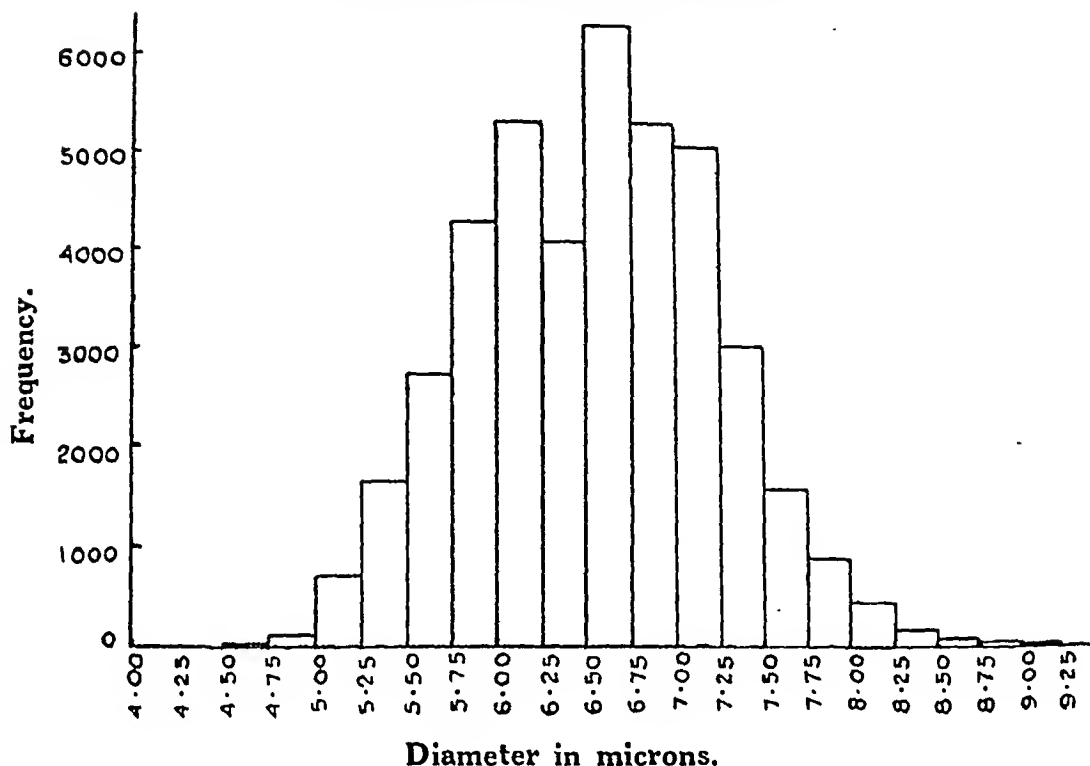
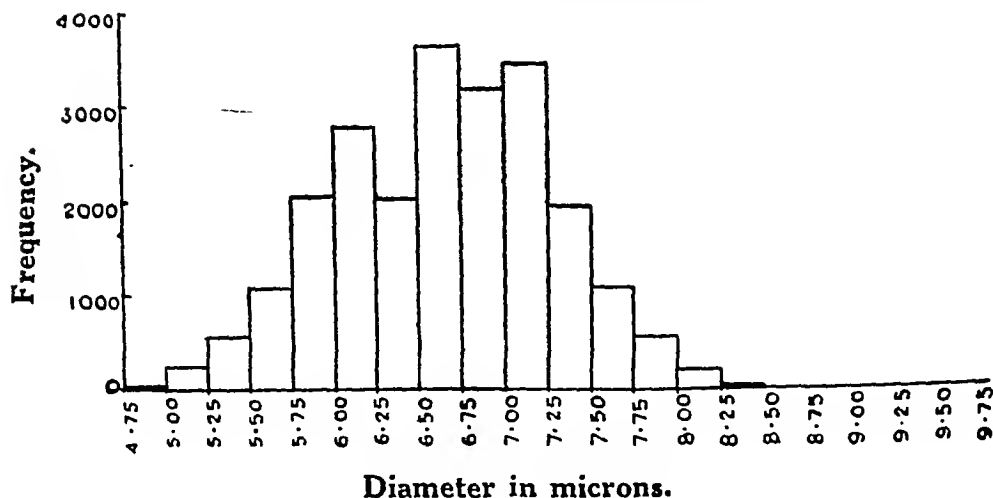


FIG. 2.

Frequency distribution of red cell diameter of 46 women of series B each contributing 500 cells.

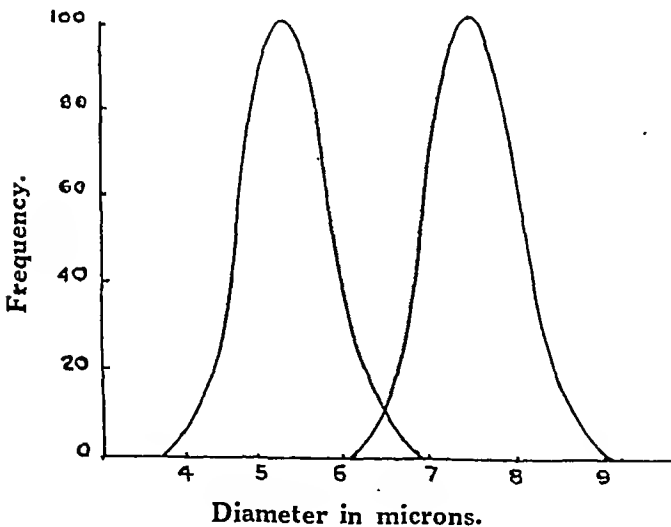


(mode to the right) with regard to skewness. Thus, while in the former case positives and negatives are evenly balanced in series B, those with negative skewness preponderate. However, the distribution of skewnesses of individual women in the two series both conform to Pearson curve type I.

Unfortunately, no other data for pregnant women are available for constructing Price-Jones' ideal curves for purpose of comparison. Since the women in series A were clinically normal and also because no regular and significant change in cell diameter can be detected in the course of pregnancy it may be justifiable to treat

FIG. 3.

*Price-Jones' maximum and minimum curves based
on the data of pregnant women
of series A.*



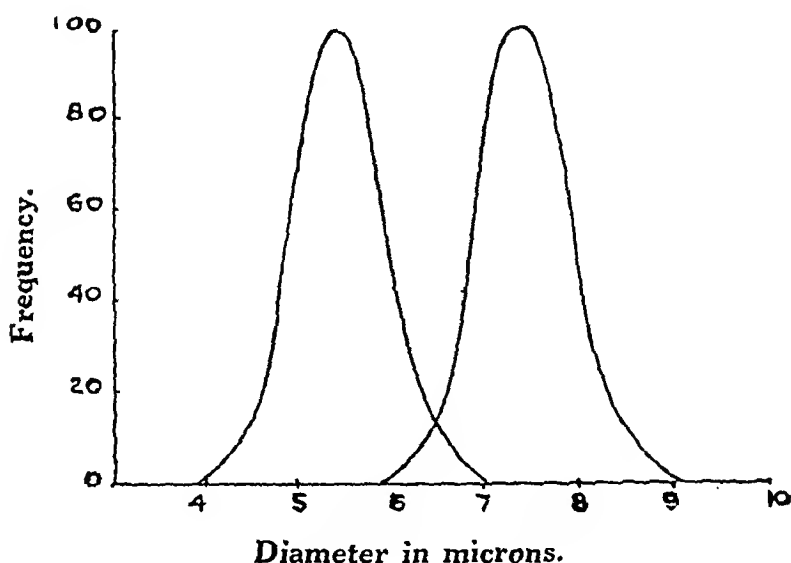
the results obtained in these series as standards for the healthy women in Calcutta, and against this series B may be compared.

The respective means of the series A and B are 6.43 ± 0.53 and 6.53 ± 0.52 which range from 5.14 to 7.72 in the former case and 5.33 to 7.73 in the latter case. Applying 't' test the difference between the means is significant. Dispersions in either case being more than 0.5 indicate certain amount of anisocytosis. The degree of variation amongst the size of individual cells of each person in series A

and B are given in *Appendix V*. Taking the limit of 7 per cent of coefficient of variation for anisocytosis, 75 per cent of individuals in series A and 78 per cent of individuals in series B would be considered as suffering from anisocytosis. The normal curves round the minimum and maximum values of the two series have been calculated and the distributions are presented in Table VIII and Figs. 3 and 4. It is obvious that series B is fully covered (except one case outside the maximum limit) by series A and therefore the two series may be considered homogeneous or in other words series B conforms to the values of the available standard series. How the standard series is different from the data for men presented by Napier

FIG. 4.

*Price-Jones' maximum and minimum curves based
on the data of pregnant women
of series B.*



et al. (1939) and Napier, Sen Gupta and Chandra Sekar (1941) for Bengalee Hindus mostly consisting of doctors and laboratory assistants may be seen from Table VIII.

Comparing the actual distribution for 42,000 red cells of the normal pregnant women of the present series with the minimum and maximum curves given by Napier *et al.* (1939), Napier, Sen Gupta and Chandra Sekar (*loc. cit.*) and Price-Jones (1933) the percentage of cells falling outside (all towards small diameter cells) are 3.7, 23.1 and 7.3 respectively.

TABLE VIII.

Comparison of the cell diameter of the present series A and B, with those recorded for healthy males by other workers.

Class intervals of r.b.c. diameter.	Series A.		Series B.		Napier <i>et al.</i> (1939).		Napier, Sen Gupta and Chandra Sekar (1941).	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
—3·375	1
—3·625	2
—3·875	7
—4·125	18	...	2
—4·375	37	...	5
—4·625	63	...	15
—4·875	85	...	33
—5·125	93	...	59	...	1
—5·375	82	...	83	...	3
—5·625	60	1	95	...	10	...	2	...
—5·875	30	2	86	1	28	...	6	...
—6·125	15	5	62	4	58	...	17	...
—6·375	5	15	36	13	89	1	39	1
—6·625	2	32	16	29	105	2	68	4
—6·875	...	57	6	54	94	9	93	14
—7·125	...	81	2	80	63	25	99	32
—7·375	...	93	...	94	32	53	82	61
—7·625	...	85	...	89	12	85	53	88
—7·875	...	63	...	67	4	105	27	100
—8·125	...	38	...	40	1	97	10	88
—8·375	...	18	...	19	...	68	3	61
—8·625	...	7	...	7	...	36	1	32
—8·875	...	2	...	2	...	14	...	14
—9·125	...	1	...	1	...	4	...	4
—9·375	1	...	1
Mean ...	6·43		6·53		7·29		7·34	
Range ...	5·14 to 7·72		5·33 to 7·73		6·64 to 7·93		6·94 to 7·75	
Coefficient of variation.	8·4		8·1		6·4		6·7	

It will be interesting to see whether the general shift towards small cells and a certain amount of anisocytosis observed amongst the normal healthy pregnant women in Calcutta as compared with healthy men in England and in Calcutta can be reduced by routine administration of iron and whether the women will benefit by it in their general health and sense of comfort.

The comparison suffers from the fact that lumping together of heterogeneous individuals in regard to their cell diameter masks the extent of microcytosis in different persons. In Table IX the individuals of series A and B are distributed according to the degree of microcytosis as determined by the percentage of cells falling to the left of the minimum ideal curve of Price-Jones.

TABLE IX.

Distribution of women in series A and B, according to the percentage of cells falling to the left of Price-Jones' minimum ideal curve.

Percentage of microcytosis.	Nil.	-5	-10	-15	-20	-25	-30	-35	-40	-45	-50
SERIES A.—											
Number of women	19	2	3	5	5	6	15	4	10	6	9
Percentage ...	22·6	2·4	3·6	5·9	5·9	7·1	17·8	4·8	11·9	7·1	10·9
SERIES B.—											
Number of women	13	5	3	5	3	2	4	1	2	3	5
Percentage ...	28·3	10·9	6·5	10·9	6·5	4·3	8·7	2·2	4·3	6·5	10·9

Thus, the degree of microcytosis is well marked in a large number of instances in both groups of the present series. In no case did any frequency curve lie to the right of the maximum ideal curve.

Results of biochemical examinations.—The results of biochemical investigations reported here refer to the normal group only. The blood volume increases in pregnancy due to increased water content. Since we have no information regarding the percentage increase which takes place amongst pregnant women in India at different periods of gestation, no attempt at correction of calcium, phosphorus and cholesterol contents have been made.

TABLE X.

Calcium in mg. per 100 c.c. of blood.

	Month of gestation.						
	-4	4-5	5-6	6-7	7-8	8-9	9-10
Number of cases ...	12	13	11	12	14	14	20
Calcium (average) {	10·38 ±0·57	9·92 ±0·61	9·85 ±0·74	9·94 ±0·84	10·03 ±0·16	9·40 ±1·96	9·77 ±0·72

Blood calcium normally varies between 9 mg. and 11 mg. per 100 c.c. It would appear that calcium contents of the blood of normal Indian women remains within the normal range throughout the period of gestation. Actually the values should have been somewhat higher than those given in the table. However, it does not necessarily signify that there is calcium deficiency for any calcium demand which

is not met by food rich in easily absorbable calcium must be supplied at the expense of mothers' teeth and bones.

TABLE XI.

Phosphorus in mg. per 100 c.c. of blood.

	Month of gestation.						
	-4	4-5	5-6	6-7	7-8	8-9	9-10
Total number of cases ...	11	12	11	12	13	12	21
Phosphorus (average) {	4.02 ±0.99	3.84 ±1.08	3.66 ±1.01	4.14 ±1.03	3.43 ±0.44	3.83 ±1.00	3.81 ±0.60

Blood phosphorus.

The normal range of blood phosphorus is 3 mg. to 5 mg. In this series blood phosphorus was estimated for 92 women. The results are shown in Table XI. The normal range is 3 mg. to 5 mg. per 100 c.c. It will be seen from Table XI that phosphorous content of the blood is maintained within normal limits throughout pregnancy. Here again it is possible that when the diet is deficient the phosphorus demand is met by the bones in some cases.

TABLE XII.

Cholesterol in mg. per 100 c.c. of blood.

	Month of gestation.						
	-4	4-5	5-6	6-7	7-8	8-9	9-10
Number of cases ...	13	9	7	12	15	14	11
Cholesterol (average) {	213.91 ±53.98	225.67 ±63.03	264.43 ±47.95	227.68 ±74.56	255.19 ±61.82	215.89 ±74.81	291.39 ±215.20

Blood cholesterol.

The cholesterol values of blood at different periods of gestation are shown in Table XII. There is some difficulty in ascertaining the normal range of value for serum cholesterol; it may be taken as 180 mg. to 200 mg. for the non-pregnant women. In a few cases the value of 180 mg. is not attained. In one instance (between 24th and 28th weeks) only 87.5 mg. were found. However, on the whole a hypercholesteræmia developing early in pregnancy and persisting throughout the period of pregnancy was observed. The mean value at term was 291.39 mg. which was the highest recorded.

*APPENDIX I.**Social conditions of the population studied.*

Blood picture of a people will necessarily be influenced by a variety of socio-economic factors the exact significance of each of which may not be easily determined. We have in the course of our investigation collected some information regarding these factors, but our data being small we are not in a position to standardize conditions with regard to all factors but one at a time, so as to isolate its influence in determining the nature of blood picture. However, it should give some idea to the reader as to the type of population we are dealing with, if a general description of the community from which our cases were drawn is given. This note is considered necessary because we wish to make it clear that in describing the socio-economic conditions we have no intention of correlating any factor with any aspect of blood picture. The discussion is limited to the women forming the 'combined normal'. Of the 746 women constituting this group, the socio-economic schedule for 31 were inadvertently missed and, therefore, the discussion relates to 715 cases. Even so, part of the information of certain individuals has been missed at the time of taking the history. In such cases the distribution will not add up to 715. However, it is felt that with all the discrepancies the discussion that follows will convey a fair idea of the socio-economic conditions of the community under study.

Economic status.

The determination of the economic status of a family presents difficulties. Accurate information about per head income in the family, whatever merit such an information may possess, is generally not obtainable; for the women either do not possess correct information or are unwilling to divulge it and some may deliberately offer misleading information for a variety of reasons. In view of these circumstances an indirect method was employed as shown in Table A. By this means it is at least possible to get a fair idea of their standard of living which after all is of greater importance than the bank balance.

TABLE A.

Distribution of women by 'economic status'.

	Cabin.	Paying.	Free.
Numbers 	120	181	387

Assuming that better accommodation was available on payment, Table A shows that more than half of the women could not, or preferred not to, spend money on hospital accommodation. Only a little more than 25 per cent availed non-paying beds. It should, however, be remembered that cabin accommodation is not provided in all the hospitals and in some even paying beds are not available.

TABLE B.

Distribution of women according to province of origin.

Province.	Bengal.	Assam.	U.P.	C.P.	Punjab and N.-W.F.P.	Bombay and Sind.	Bihar.	Orissa.	Rajputana.	Madras and Hyderabad.	Gujrat.	Others.
Numbers ...	347	6	47	4	63	25	83	5	54	18	41	16
Percentage ...	48.9	0.8	6.6	0.6	8.9	3.5	11.7	0.7	7.6	2.6	5.8	2.3

Calcutta, like other large coastal towns, is cosmopolitan and the population is heterogeneous with regard to the province of origin. On account of the gregarious habits of the people of common origin, different sections of the city are predominantly inhabited by persons hailing from certain provinces and the hospital habit is also unequally developed in different sections of the town. For these reasons the sample investigated is not necessarily representative of the Calcutta population in respect of the province of origin. Table B gives the numbers and percentage of persons coming from the various provinces. Naturally, the largest numbers of women belong to Bengal (about 50 per cent) and about 12 per cent to the neighbouring province of Bihar. The Punjab and the N.-W.F. Province are also well represented. Next in importance are the U.P. and Rajputana. The low representation of the neighbouring provinces of Orissa, Assam and the C.P. support our previous remark that the sample is not representative of the Calcutta population. The relatively high proportion of the Punjabees and people from Rajputana, as also from Gujrat, shows that commercial communities which are rather well off are likely to be over represented. This fact must be borne in mind in interpreting the results inasmuch as anæmia in pregnancies is believed to be correlated with economic condition.

TABLE C.

Distribution of the women according to occupation.

	Housewife.	Manual worker.	Clerk.	Professional.
Numbers ...	659	36	3	2
Percentage ...	94.1	5.1	0.4	0.3

The number of women occupied in gainful occupation is low in Bengal. According to 1931 Census the percentage of 'active' women in Bengal constituted only 7 per cent of the women population against 28 per cent in India as a whole. In a recent study in a rural area in Bengal by Mathen and Lal (1946) even at the time of stress when inducements and necessities for employment would be abnormally high only 3 per cent of females were found 'active'. It is not known what proportion of the female population would be normally 'active' under urban conditions. It may be noted that our sample of normal women includes significant proportion of poor women who have to earn their living by manual work (*see* Table C). Whether or not this proportion truly represents the Calcutta population is difficult to say, but it must be remembered that a majority of manual workers constitutes floating population and they generally leave their families behind.

TABLE D.

Distribution of women according to previous history of anaemia.

	Yes.	No.	TOTAL.
Numbers ...	140	491	631
Percentage ...	22·2	77·8	100

Record of the history of anæmia on some previous occasion amongst women who now present normal blood picture is available for 631; of these 22·2 per cent were able to recall the existence of anæmia on some previous occasion (*see* Table D). Anæmia is a condition of which subjective symptoms may not necessarily be severe enough to be noted and remembered by common women and it is, therefore, likely that a higher proportion experienced anæmic conditions in previous pregnancies than this.

At any rate the point of interest lies in that about one-fourth and possibly more of the pregnant women, who were in clinically normal condition at the time of investigation, had had anæmia on some previous occasion. From the information given by 66 women (*see* Table F) it would appear that the condition is most frequently noticed during the hot pre-monsoon months, viz. May and June.

TABLE E.

Distribution of women by month of onset of anaemia.

Months :—	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Numbers ...	8	2	2	7	11	9	5	5	8	2	5	2

TABLE F.

Distribution of women according to diet.

Staple food :—	RICE.			WHEAT.			MIXED.		
	Vegetarian.	Fish.	Fish and meat.	Vegetarian.	Fish.	Fish and meat.	Vegetarian.	Fish.	Fish and meat.
Frequency ...	36	120	68	30	182	28	12	106	128

Majority of people take wheat and mixed rice and wheat diet (see Table F). Pure rice-eaters are in minority. All but 78 women had no objection to fish or meat and about one-third ate these articles regularly. Over 93 per cent of women get some milk but majority of them do so only occasionally. Information about the quantity of milk is not satisfactory. Of the women for whom information is available 71·4 per cent take ghee or clarified butter but in most cases this article of food is available only occasionally.

TABLE G.

Distribution by religion.

Religion :—	Hindus.	Muslims.	Christians.	Sikhs.	Others.	TOTAL.
Numbers ...	527	170	11	1	2	711
Percentage ...	74·1	23·9	1·5	0·1	0·3	...

Hindus formed the bulk of the sample, only 23·9 per cent were Muslims. Christians and others were very few in number (see Table G). This is almost the correct denominational proportion in Calcutta population.

TABLE H.

Distribution of women by age.

Age :—	—13	13—14	14—15	15—20	20—25	25—30	30—35	35—40	40—45	TOTAL.
Numbers ...	1	1	9	154	222	158	123	32	9	709
Percentage ...	0·1	0·1	1·3	21·7	31·3	22·3	17·3	4·5	1·3	...

A little less than one-third of the women belonged to the age group 20 to 25 years; over three-fourths were between the ages 15 and 30, 1·5 per cent were below 15 and the rest above 30 (see Table H).

APPENDIX II.

Dr. S. Mitra's cards regarding anæmia and pregnancy inquiry.

General case schedule (clinical 'A').

Name..... Register number.....

Clinician.....

Hospital.....

Year.....

I, II, III, IV. *Serial number*.....V. *Province of origin*.—Bengal. Assam. U.P. C.P. Punjab & N.-W.F. P. Bombay & Sind.
0 1 2 3 4 5Bihar. Orissa. Rajputana. Madras & Hyderabad.
6 7 8 9VI. *Month of admission*.—Jan. Feb. Mar. Apr. May. June. July. Aug. Sep. Oct. Nov. Dec.
1 2 3 4 5 6 7 8 9 10 11 12VII. *Age*.—13, 13-14, 14-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-.
0 1 2 3 4 5 6 7 8 9VIII. *Religion*.—Hindu. Muslim. Christian. Sikh. Others.
0 1 2 3 4IX. *Occupation*.—Housewife. Manual worker. Clerical. Professional. Others.
0 1 2 3 4X. *Economic status*.—Cabin. Paying bed. Free.
0 1 2XI, XII. *Duration of marriage (in years)*.....XIII. *Month of pregnancy*.—3, 3-, 4-, 5-, 6-, 7-, 8-, 9-.
0 1 2 3 4 5 6 7XIV. *Previous pregnancy (exact number to be punched)*.XV. *Menstrual history, previous to pregnancy*.—Scanty. Moderate. Profuse. Regular.
0 1 2 3
Irregular. Clots. Pain.
4 5 6XVI. *Previous history of anæmia*.—Yes. No.
0 1XVII. *Month of onset of anæmia*.—Jan. Feb. Mar. Apr. May. June. July. Aug. Sep. Oct. Nov.
1 2 3 4 5 6 7 8 9 10 11
Dec.
12XVIII. *Diet*.—R1. R2. R3. W1. W2. W3. M1. M2. M3. Chana.
0 1 2 3 4 5 6 7 8 9XIX. *Milk*.—Occasional. Regular. -1st pau. 1- $\frac{1}{2}$ sr. $\frac{1}{2}$ sr. 1 sr-
0 1 2 3 4 5*Ghee*.—Yes. No.
6 7

- XX. Disease (*T. B. clinically*).—Nil. Early. Late.
0 1 2
- XXI. Chronic malaria.—No. Chronic. Acute.
0 1 2
- XXII. Health (*general look*).—Well-nourished. Moderately-nourished. Badly-nourished. Pale.
0 1 2 3
- XXIII. Appetite.—Good. Indifferent. Bad.
P 0 1 2
U 3 4 5
- XXIV. Bowel movement.—Once. Twice. More than twice. Once in two or more days.
P 0 1 2 3
U 4 5 6 7
- XXV. Liver.—No path. Enlarged. Other condition.
0 1 2
- Spleen.—Not enlarged. Enlarged. Palp. Two fingers. More than two fingers.
3 4 5 6 7
- XXVI. Intestine.—No path. Tenderness in caecal region. Thickening in caecal region.
0 1 2
- Tenderness in sigmoidal region. Thickening in sigmoidal region.
3 4
- XXVII. Heart.
- XXVIII. Treatment with iron or liver.

Anæmia and pregnancy inquiry.

General case schedule (laboratory card).

Name.....

- I, II, III, IV. Serial number.....
- V. R.b.c. (in millions).—1, 1-, 1·5, 2, 2·25, 2·5, 3, 3·5, 4, 4·5, 5.
0 1 2 3 4 5 6 7 8 9 10
- VI. W.b.c. (in thousands).—2-, 3-, 4-, 5-, 6-, 9-, 10-, 12-, 16-, 24-.
0 1 2 3 4 5 6 7 8 9
- VII. Reticulocytes, per cent.—0·2, 0·5, 0·8, 1-, 2-, 3-, 5-, 8-, 10-, 15-.
0 1 2 3 4 5 6 7 8 9
- VIII. Hæmoglobin (Sahli), per cent.—10, 10-, 15, 20, 25, 35, 45, 55, 65, 75, 85+.
0 1 2 3 4 5 6 7 8 9 10
- IX. Hæmoglobin Pulfrich, per cent.—10, 10-, 15-, 20, 25, 35, 45, 55, 65, 75, 85+.
0 1 2 3 4 5 6 7 8 9 10
- X. Hæmoglobin, grammes per 100 c.c.—2-, 4-, 6-, 8-, 10-, 11-, 12, 13, 14, 15+.
0 1 2 3 4 5 6 7 8 9
- XI. Mean corpuscular volumes.—55, 60-, 70-, 79-, 94-, 98-, 100, 110, 120, 140, 160.
0 1 2 3 4 5 6 7 8 9 10
- XII. Cell volumes.—10, 10-, 20-, 30-, 35-, 40-, 45-, 50-, 55-, 60-.
0 1 2 3 4 5 6 7 8 9
- XIII. M.c.h.—10-, 15-, 22-, 27-, 30-, 35-, 40-, 45-, 50-.
0 1 2 3 4 5 6 7 8
- XIV. M.c.h. concentrates.—15-, 20-, 22-, 25-, 29-, 30-, 33-, 35-, 38-, 40.
0 1 2 3 4 5 6 7 8 9

XV.	Colour index.—	-0.4,	0.4-	0.5-	0.6-	0.7-	0.8-	0.9-	1.0-	1.2-	1.5-					
		0	1	2	3	4	5	6	7	8	9					
XVI.	Abnormal cell.—	Megaloblast.	Normoblast.	Polychromasia.	Stippling.	Türk cell.										
		0	1	2	3	4										
		Microcytes.	Myeloblasts.	Basophils.	Poikilocytosis.	Anisocytosis.										
		5	6	7	8	9										
	Vacuolation.															
		10														
XVII.	Differential count.	P.C.—	Neutrophils.	Basophils.	Eosinophils.	Monocytes.										
		D.C.	0	1	2	3										
			Lymphocytes, large.	Lymphocytes, small.	Granulocytes, toxic.											
			4	5	6											
			Lymphocytes, abnormal.													
			7													
XVIII.	Differential count per c.mm.—	Neutrophils.	Basophils.	Eosinophils.	Monocytes.											
		0	1	2	3											
			Lymphocytes, large.	Lymphocytes, small.												
			4	5												
			Granulocytes, toxic.	Lymphocytes, abnormal.												
			6	7												
XIX.	Platelet count.—	-200,000.	200,000-.	500,000-.	600,000-.	800,000+.										
		0	1	2	3	4										
XX.	Van den Bergh reaction.—	Negative.	Direct.	Indirect.												
		5	6	7												
XXI.	Icterus index.—	-5,	5-	7.5,	10,	15-	20.									
		0	1	2	3	4	5									
XXII.	Sedimentation.—	Rapid.	Slow.	Normal.												
		6	7	8												
XXIII.	Urine.—	Albumen	Sugar.	By Fehling's solution.	By Nylander's solution.											
		Yes.	No.	Yes.	No.	Yes.	No.									
		0	1	2	3	4	5									
XXIV.	Cast.	Hyaline.	Granular.	Othor.	Nil.											
		6	7	8	9	10										
XXV.	Kahn test	Positive.—	+++	++	+											
		Negative.	0	1	2											
			3													
XXVI.	Ankylostomiasis.—	Yes.	No.													
		4	5													
XXVII.	Malarial parasites.—	Yes.	No.	Pigment.												
		6	7	8												
XXVIII.	Mean cell diameter.—	-5.50,	5.50-	5.8-	6.0-	6.5-	6.7-	7.0-	7.5-	8.0-	8.5-	9.0-				
		0	1	2	3	4	5	6	7	8	9	10				
		9.5-	9.6-	9.8-												
		11	12	13												
XXIX.	Coefficient variation.—	4%	5,	6,	7,	8,	9,	10,	11,	12.						
		0	1	2	3	4	5	6	7	8						
XXX.	Standard deviation.—	0.3,	0.4,	0.5-	0.6,	0.7,	0.8,	0.9,	1.0,	1.1,	1.2,	1.3,	1.4,	1.5,	1.6,	1.7,
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
		1.8,	1.9-													
		15	16													

APPENDIX III.

Technique employed for biochemical estimation.

Blood calcium.—Two c.c. of clear serum were introduced into a graduated 15 c.c. centrifuge tube; 2 c.c. of distilled water and 1 c.c. of 4 per cent ammonium oxalate solution were added and the contents were mixed thoroughly. After allowing the tube to stand for 2 hours or more the contents were again mixed and centrifuged for 5 minutes at 2,500 r.p.m. After syphoning off the supernatant fluid, the calcium precipitate was broken up with about 2 c.c. of 2 per cent ammonia directed in a fine jet. A further 3 c.c. of ammonia were used to wash down the sides of the tube. Centrifuging was then repeated for 3 minutes at 2,500 r.p.m. and the fluid was drained off as before. This process was repeated for the third time.

Two c.c. of normal sulphuric acid was added to the washed precipitate, the tube was placed in a water-bath at 100°C. and the contents were shaken gently at intervals till the precipitate was dissolved. Titration was done before cooling (as rapidly as consistent with accuracy) with N/100 KMnO_4 till a definite pink colour was obtained which persisted for at least one minute.

Inorganic phosphorus in blood.—Two c.c. of clear serum were blown into 6 c.c. of 13.3 per cent trichlor-acetic acid in a test-tube. The pipette was filled with water which was allowed to empty slowly into the test-tube. It was shaken vigorously and allowed to stand for a few minutes. The fluid was filtered, 1.25 c.c. of the filtrate were pipetted into a test-tube to which 1.15 c.c. of molybdate II (dil.) was added and the tube was rotated between the fingers to mix. 0.1 c.c. of amino-naphthol-sulphonic acid was then added and the tube inverted once to mix. To prepare the standard, 5 c.c. of standard phosphate solution, 70 c.c. water, and 10 c.c. molybdate I were put into a 100 c.c. flask and mixed well. To this 4 c.c. of 1 : 2 : 3 : sulphonic acid were added, diluted with distilled water to the mark 1, mixed well and measured colorimetrically after 10 minutes.

Free and combined cholesterol:

(a) *Extraction from serum.*—15 c.c. of acetone-alcohol solution were heated to boiling in a 25-c.c. measuring flask on a water-bath, 1 c.c. of serum was injected into the hot solution with a pipette, the liquid being kept constantly stirred. The flask was then closed and vigorously shaken to break the lumps of precipitated albumen. The stopper was removed and washed and the flask again heated to boiling point in the water-bath. After cooling to room temperature, it was filled up to the mark 25 c.c. with acetone alcohol, shaken and filtered through a clean dry filter.

(b) *Precipitation of free cholesterol.*—Two c.c. of the clean filtrate were mixed with 1 c.c. of digitonin solution in a conical 15 c.c. centrifuge tube and well stirred with a glass-rod. The centrifuge tube and the glass-rod were allowed to stand overnight in a closed half-litre vessel at room temperature. Next morning, the centrifuge tube was removed from the vessel and the contents carefully swirled round to free any portions of the precipitate which might be clinging to the walls.

The glass-rod was then removed from the centrifuge tube without touching the upper part of the tube and carefully laid on a clean stand so that no adhering precipitate was lost. The tube was then centrifuged at 2,500 r.p.m. for 15 minutes. The clear liquid was carefully drawn off through a fine capillary tube with a suction pump without touching the wall of the tube with the capillary tube or stirring up the precipitate.

The glass-rod was then put back in the centrifuge tube and it and the walls of the centrifuge tube were rinsed down with 2 c.c. of acetone-ether solution from a dropping pipette. After thorough mixing, the glass-rod was removed, observing the same precaution as before and the tube was centrifuged for 5 minutes. Greater care was taken this time in drawing off the clear liquid. The precipitate was washed twice again in the same manner with pure ether, and after the last washing when the fluid had been removed, the centrifuge tube together with the glass-rod was placed in a water-bath at 40°C. The precipitate was dried in about 2 to 3 minutes. The sample was then ready for colour development.

(c) *Precipitation of total cholesterolin.*—One c.c. of serum extract was pipetted into a 15 c.c. centrifuge tube, a drop of caustic potash solution was added and well mixed with a glass-rod (the drop of KOH was of such size that about 0.1 c.c. of 5 per cent HCl was needed to neutralize it). The glass-rod was left in the centrifuge tube. The tube was then placed on a stand in a water-bath at 40°C. for half an hour to complete the hydrolysis.

After it had cooled, 1 c.c. of acetone-alcohol solution was added. The liquid was carefully neutralized with 5 per cent HCl using phenolphthalin as indicator. The solution was thoroughly mixed as each drop of HCl was added (an alkaline reaction of the liquid was carefully avoided at the end). One c.c. of digitonin solution was added to the centrifuge tube which was allowed to stand overnight to ensure complete precipitation. The precipitate was centrifuged off and treated as described for the isolation of free cholesterolin.

(d) *Colour reaction and photometric measurement.*—The dried precipitate of cholesterolin-digitonin was now dissolved in 1 c.c. of acetic acid, the walls of the centrifuge tube and the glass-rod were rinsed down with the acid. Solution was accelerated by heating to 60°C. in a water-bath and by shaking. When the precipitate was completely dissolved, the sample was placed in a second bath at 25°C.

Two c.c. of acetic anhydride and 0.1 c.c. of concentrated sulphuric acid were added and steadily stirred with glass-rod which was still in the tube. For complete development of colour, the bath was kept at 25°C. for 27 to 37 minutes. The photometric measurement was made with filter S61 using the micro-cells of 50-mm. stratum length. The cell in the comparison beam was filled with a compensating liquid consisting of 1 c.c. acetic acid, 2 c.c. acetic anhydride and 0.1 c.c. concentrated sulphuric acid.

APPENDIX IV.

Average blood picture of the various groups of pregnant women.

Group.	1	2	3	4	5
Number of women	359	20	132	151	15
R.b.c. (in millions)	4.17 ± 0.56	4.15 ± 0.60	4.05 ± 0.48	4.18 ± 0.62	4.42 ± 0.45
W.b.c. (in thousands)	8.48 ± 2.58	8.62 ± 2.48	8.14 ± 2.34	8.42 ± 2.82	9.60 ± 3.60
Retiocyte, per cent	0.63 ± 0.55	0.57 ± 0.37	0.72 ± 0.73	0.72 ± 0.71	0.50 ± 0.32
Hemoglobin, per cent	10.51 ± 1.56	10.60 ± 1.34	10.19 ± 1.38	10.40 ± 1.68	10.70 ± 1.67
Mean corpuscular volume	81.30 ± 7.87	82.98 ± 9.53	80.07 ± 7.84	81.02 ± 9.51	79.63 ± 7.8
Cell volume	32.77 ± 5.06	34.00 ± 4.55	31.64 ± 5.02	32.97 ± 5.38	34.00 ± 4.55
Mean corpuscular hemoglobin	25.29 ± 4.4	26.00 ± 3.15	25.06 ± 2.86	25.10 ± 3.16	23.17 ± 2.98
Mean corpuscular hemoglobin content.	31.61 ± 2.5	31.15 ± 2.7	31.94 ± 2.5	31.27 ± 2.53	30.93 ± 3.42
Colour index	0.97 ± 0.17	0.99 ± 0.17	0.97 ± 0.18	0.96 ± 0.17	0.89 ± 0.11
Platelet count (in thousands)	200.0 ± 136.5	215.0 ± 158.2	178.6 ± 121.2	180.0 ± 111.2	116.7 ± 64.5
Icterus index	7.0 ± 2.3	6.7 ± 2.2	6.6 ± 2.4	7.3 ± 2.1	7.3 ± 1.8
Neutrophil count	6088.1 ± 2569.0	6200.0 ± 1614.4	5943.4 ± 1791.0	6054.5 ± 1927.0	6883.0 ± 1831.9
Eosinophil count	157.2 ± 906.8	216.2 ± 278.9	204.5 ± 257.8	129.3 ± 180.0	186.7 ± 229.5
Monocyte count	148.3 ± 238.6	171.2 ± 283.7	147.1 ± 258.9	199.4 ± 327.9	116.7
Lymphocyte (large) count	477.7 ± 408.9	625.0 ± 531.5	468.8 ± 365.1	487.2 ± 492.0	520.0 ± 502.4
Lymphocyte (small) count	1238.3 ± 700.1	1247.5 ± 691.4	1146.9 ± 683.0	1192.2 ± 773.5	1401.7 ± 766.8

APPENDIX IV—contd.

Group.	6	7	8	9	10	11
Number of women	65	41	28	75	43	75
R.b.c. (in millions)	3.99 ± 0.58	4.18 ± 0.58	3.73 ± 0.90	3.99 ± 0.78	3.84 ± 0.58	3.84 ± 0.82
W.b.c. (in thousands)	8.20 ± 2.40	7.85 ± 1.99	8.46 ± 1.58	8.35 ± 2.24	7.36 ± 1.90	8.68 ± 7.74
Reticulocyte, per cent	0.79 ± 0.60	0.69 ± 0.67	1.12 ± 1.04	0.99 ± 0.99	1.33 ± 1.97	1.02 ± 0.80
Hæmoglobin, per cent	9.99 ± 1.49	10.56 ± 1.55	9.52 ± 1.82	10.20 ± 1.96	10.08 ± 1.58	9.73 ± 2.11
Mean corpuscular volume	81.92 ± 9.64	81.44 ± 9.90	82.21 ± 7.03	80.38 ± 8.18	81.36 ± 7.80	81.35 ± 8.14
Cell volume	31.67 ± 4.62	32.55 ± 4.88	29.91 ± 5.75	31.31 ± 5.90	30.29 ± 5.27	30.70 ± 7.0
Mean corpuscular hæmoglobin	24.96 ± 3.48	24.18 ± 4.00	25.21 ± 4.26	25.39 ± 5.47	25.76 ± 4.72	25.18 ± 4.63
Mean corpuscular hæmoglobin concent.	31.47 ± 2.47	32.23 ± 2.03	31.73 ± 1.79	31.85 ± 2.60	32.02 ± 1.72	31.89 ± 2.33
Colour index	0.96 ± 0.13	0.96 ± 0.17	0.96 ± 0.19	0.99 ± 0.23	1.05 ± 0.26	0.99 ± 0.20
Platelet count, (in thousands)	157.4 ± 106.0	213.3 ± 138.7	189.3 ± 122.0	178.1 ± 116.8	175.0 ± 116.0	211.5 ± 125.1
Icterus index	7.1 ± 2.5	7.1 ± 2.1	7.7 ± 1.2	8.4 ± 2.8	8.6 ± 2.8	8.5 ± 2.6
Neutrophil count	5926.2 ± 255.0	6000.0 ± 1674.7	6125.0 ± 1504.1	6148.4 ± 2284.0	5273.0 ± 1771.8	6180.0 ± 2245.4
Eosinophil count	173.8 ± 242.4	126.5 ± 176.5	236.6 ± 216.5	159.4 ± 215.6	181.4 ± 183.8	133.0 ± 193.8
Monocyte count	165.6 ± 347.6	198.0 ± 253.7	165.2 ± 353.7	172.7 ± 307.2	137.2 ± 207.0	98.3 ± 203.9
Lymphocyte (large) count	468.9 ± 388.7	515.3 ± 435.0	383.7 ± 384.8	430.7 ± 379.5	395.3 ± 333.2	368.0 ± 340.0
Lymphocyte (small) count	1209.4 ± 642.1	1195.4 ± 690.6	1413.4 ± 648.0	1159.3 ± 633.4	1023.3 ± 545.1	1290.0 ± 637.3

APPENDIX IV—concl'd.

Group.	12	13	14	15	16	17
Number of women	31	10	16	27	15	27
R.b.c. (in millions)	4.12 ± 0.50	3.90 ± 0.40	4.30 ± 0.57	4.23 ± 0.82	3.51 ± 0.61	3.97 ± 0.62
W.b.c. (in thousands)	8.97 ± 2.72	7.72 ± 1.20	8.50 ± 1.47	8.37 ± 1.05	7.37 ± 1.31	7.14 ± 1.79
Retiucytes, per cent	0.90 ± 0.73	0.90 ± 0.53	0.74 ± 0.16	1.08 ± 0.94	1.23 ± 0.70	1.01 ± 0.97
Hæmoglobin, per cent	10.21 ± 1.78	9.85 ± 1.50	10.32 ± 2.06	10.12 ± 1.76	9.37 ± 1.63	9.88 ± 1.78
Mean corpuscular volume	89.31 ± 7.14	77.40 ± 8.64	86.70 ± 9.42	77.35 ± 8.77	87.73 ± 12.50	82.90 ± 12.02
Cell volume	31.51 ± 5.59	28.75 ± 3.95	32.05 ± 5.90	31.54 ± 4.90	28.50 ± 3.87	31.00 ± 6.13
Mean corpuscular hæmoglobin	24.85 ± 2.81	22.90 ± 4.09	21.05 ± 3.70	23.73 ± 4.08	26.03 ± 6.55	23.06 ± 4.64
Mean corpuscular hæmoglobin concent.	31.42 ± 2.91	31.90 ± 2.31	31.14 ± 2.54	31.79 ± 2.75	31.13 ± 2.16	30.78 ± 2.93
Colour index	0.97 ± 0.22	1.00 ± 0.22	0.92 ± 0.28	0.90 ± 0.10	1.02 ± 0.13	0.97 ± 0.18
Platelet count (in thousands)	192.1 ± 122.2	150.0 ± 33.3	190.9 ± 126.1	194.7 ± 157.2	200.0 ± 136.8	196.0 ± 123.2
Icterus index	7.8 ± 1.9	7.6 ± 2.1	7.1 ± 2.0	7.3 ± 1.9	7.0 ± 2.5	7.6 ± 2.2
Neutrophil count	6309.2 ± 1654.0	6450.0 ± 1770.8	6000.0 ± 444.4	5721.0 ± 1416.6	5200.0 ± 2186.6	5000.0 ± 1369.2
Eosinophil count	307.9 ± 285.1	440.0 ± 279.7	222.7 ± 147.3	110.6 ± 149.4	183.3 ± 226.5	53.0 ± 85.0
Monocyte count	113.2 ± 183.7	35.0 ± 110.5	227.3 ± 191.5	193.3 ± 269.4	243.3 ± 398.9	70.0 ± 156.1
Lymphocyte (large) count	493.4 ± 375.5	445.0 ± 232.6	577.3 ± 446.3	467.3 ± 382.6	483.3 ± 348.3	466.0 ± 357.5
Lymphocyte (small) count	1157.9 ± 665.9	1210.0 ± 1317.5	1181.8 ± 2064.0	1040.4 ± 566.9	1315.0 ± 487.2	1291.0 ± 640.2

APPENDIX V.

Mean cell diameter of 500 cells of the pregnant women of series A.

Serial number.	Mean.	Standard deviation.	Coefficient of variation.
1	6.1875	0.4955	8.0
2	6.0500	0.6070	10.0
3	5.8600	0.4828	8.2
4	5.9560	0.4122	6.9
5	6.1095	0.4193	6.9
6	6.3750	0.5419	8.5
7	6.7610	0.3878	5.7
8	5.7815	0.5815	10.0
9	6.3845	0.6191	9.7
10	6.3310	0.5796	9.1
11	5.9545	0.5667	9.5
12	6.2635	0.6632	10.6
13	6.4165	0.7457	11.6
14	6.6795	0.5367	8.0
15	6.5615	0.4274	6.5
16	6.8470	0.4008	6.7
17	6.6250	0.4385	6.6
18	6.7155	0.4512	6.7
19	5.7744	0.4959	8.6
20	6.4395	0.4457	6.9
21	6.5976	0.5199	7.9
22	7.3290	0.5278	7.2
23	6.6585	0.4722	7.1
24	6.0045	0.5718	9.5
25	6.6890	0.6283	9.4
26	6.3840	0.6429	10.1

APPENDIX V—contd.

Mean cell diameter of 500 cells of the pregnant women of series A—contd.

Serial number.	Mean.	Standard deviation.	Coefficient of variation.
27	7.0245	0.5740	8.2
28	6.5780	0.7350	11.2
29	6.6420	0.7461	11.2
30	6.1255	0.5883	9.6
31	6.8360	0.5272	7.7
32	7.3845	0.5168	7.0
33	6.7310	0.4713	7.0
34	5.6700	0.3240	5.7
35	6.7700	0.4336	6.4
36	7.1230	0.5701	8.0
37	6.9150	0.4971	7.2
38	6.5720	0.5380	8.2
39	6.1805	0.6173	10.0
40	6.4710	0.6816	10.5
41	7.0360	0.5928	8.4
42	7.0280	0.4270	6.1
43	7.3695	0.6498	8.8
44	6.0485	0.5795	9.6
45	5.5350	0.5038	9.1
46	6.4035	0.5785	9.0
47	6.4195	0.5554	8.7
48	6.5355	0.4656	7.1
49	5.4105	0.4535	8.4
50	6.3285	0.5564	8.8
51	6.0810	0.4684	7.7
52	6.8875	0.5561	8.1
53	6.8535	0.5054	7.4

*APPENDIX V—contd.**Mean cell diameter of 500 cells of the pregnant women of series A—contd.*

Serial number.	Mean.	Standard deviation.	Coefficient of variation.
54	6.4910	0.6271	9.6
55	6.4830	0.4847	7.5
56	6.8700	0.5463	8.0
57	5.6960	0.4030	7.1
58	6.6575	0.6416	9.6
59	6.1020	0.5463	9.0
60	6.3705	0.4976	7.8
61	6.3655	0.5531	8.7
62	5.0140	0.6302	10.6
63	6.4180	0.4016	6.3
64	6.1450	0.6047	9.8
65	6.5010	0.4394	6.8
66	6.4105	0.4425	6.9
67	6.2995	0.3912	6.2
68	5.9810	0.3994	6.7
69	6.1040	0.5640	9.2
70	6.1960	0.5644	9.1
71	6.2180	0.5745	9.2
72	5.7180	0.4852	8.5
73	6.2125	0.4698	7.6
74	6.6010	0.4134	6.3
75	6.3890	0.4163	6.5
76	6.4910	0.4531	7.0
77	6.4030	0.4183	6.5
78	7.3315	0.5821	7.9
79	6.9590	0.4683	6.7
80	6.3435	0.6810	10.7

APPENDIX V—*contd.*

Mean cell diameter of 500 cells of the pregnant women of series A—concl'd.

Serial number.	Mean.	Standard deviation.	Coefficient of variation.
81	6.5775	0.5577	8.5
82	6.7450	0.4157	6.2
83	6.3400	0.5534	8.7
84	5.4710	0.4812	8.8

Diameter of 500 cells each of pregnant women of series B.

Serial number.	Mean value.	Standard deviation.	Coefficient of variation.
1	5.9560	0.3726	6.3
2	6.1025	0.5016	8.2
3	6.8185	0.4659	6.8
4	6.2695	0.5353	8.5
5	7.2125	0.5546	7.7
6	6.1835	0.5000	8.1
7	6.5615	0.4825	7.3
8	6.1155	0.5574	9.1
9	5.8605	0.6356	10.8
10	5.6070	0.4061	7.2
11	6.0850	0.4406	7.2
12	5.7255	0.5163	9.0
13	7.0845	0.4901	6.9
14	5.7185	0.3226	5.6
15	6.6025	0.5159	7.8

*APPENDIX V—contd.**Diameter of 500 cells each of pregnant women
of series B—contd.*

Serial number.	Mean value.	Standard deviation.	Coefficient of variation.
16	6.8105	0.5682	8.3
17	6.6845	0.5696	8.5
18	7.0665	0.6783	9.6
19	6.9510	0.4441	6.4
20	6.8915	0.5712	8.3
21	6.5490	0.5051	7.7
22	6.4700	0.6513	10.1
23	6.3335	0.5004	7.9
24	6.7370	0.4192	6.2
25	6.3845	0.5636	8.8
26	6.6170	0.4840	7.3
27	6.7765	0.4084	6.0
28	6.8335	0.5465	8.0
29	6.6710	0.4666	7.0
30	6.5690	0.5651	8.6
31	6.6840	0.4525	6.8
32	5.8915	0.4807	8.1
33	7.0265	0.4128	5.9
34	6.8035	0.4732	6.9
35	6.5275	0.5313	8.1
36	6.3725	0.5831	9.1
37	6.4550	0.5664	8.8
38	6.7935	0.5229	7.7
39	6.3845	0.6058	9.5
40	6.9010	0.5481	7.9
41	6.8215	0.5841	8.6

APPENDIX V—concl'd.

*Diameter of 500 cells each of pregnant women
of series B—concl'd.*

Serial number.	Mean value.	Standard deviation.	Coefficient of variation.
42	6.6170	0.4913	7.4
43	6.7635	0.5360	7.9
44	6.8725	0.5242	7.6
45	6.9855	0.5234	7.5
46	6.2910	0.5993	9.5

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STATISTICAL STUDIES IN GLUCOSE TOLERANCE.

Part II.

BLOOD GLUCOSE OF NORMAL FEMALE SUBJECTS.

BY

M. C. VARIYAR.

(From the Department of Biochemistry, Medical College, Madras.)

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THE values for the blood glucose estimations done on 18 healthy women students of this College under conditions identical with those reported by Variyar and Mannadi Nayar (1946), on statistical analysis, showed considerable variations especially in the fasting blood-sugar results. The tests were then repeated on another 20 women students and thereby raised the total number to 38 in order to lessen the experimental errors. The results are tabulated below:—

TABLE.

Values in females between the ages of 17 and 25 years.

Serial number.	Height in inches.	Weight in pounds.	Body-surface area in square metres.	Age in years.	Fasting blood-glucose values in mg./100 c.c.
1	54.5	96.0	1.26	22.77	76.90
2	63.6	115.8	1.53	21.55	90.70
3	59.0	106.0	1.41	20.44	83.79
4	60.4	95.0	1.36	21.50	75.85
5	60.0	87.0	1.30	20.97	76.60
6	60.3	86.0	1.30	20.55	69.76

TABLE—*contd.*

Serial number.	Height in inches.	Weight in pounds.	Body-surface area in square metres.	Age in years.	Fasting blood-glucose values in mg./100 c.c.
7	60.3	115.0	1.48	23.34	96.60
8	60.0	93.0	1.34	23.51	91.30
9	60.3	98.0	1.38	17.56	84.03
10	63.0	106.0	1.47	24.52	103.35
11	68.0	128.0	1.68	23.19	91.30
12	61.5	94.5	1.38	17.83	72.40
13	60.0	106.0	1.42	24.52	79.85
14	63.0	108.0	1.48	18.76	81.00
15	64.0	102.0	1.46	20.24	101.50
16	61.0	101.0	1.41	23.29	80.00
17	58.1	90.0	1.30	17.32	107.50
18	61.0	100.0	1.41	20.98	86.60
19	64.0	117.3	1.55	21.39	72.83
20	61.0	97.3	1.38	18.30	77.52
21	60.4	97.3	1.37	22.12	70.42
22	59.0	94.8	1.34	19.48	72.73
23	63.0	148.0	1.69	24.49	76.34
24	63.0	111.5	1.50	19.27	81.63
25	62.3	85.0	1.33	20.57	86.96
26	61.0	95.0	1.37	17.95	83.33
27	60.4	110.0	1.45	16.96	82.99
28	66.0	115.0	1.57	18.69	80.65
29	61.3	131.0	1.57	18.80	83.03
30	65.5	103.0	1.50	23.03	69.69
31	60.0	80.0	1.25	20.07	80.65
32	59.8	92.0	1.33	19.77	69.93
33	60.3	96.0	1.36	18.18	70.67

TABLE—*concl'd.*

Serial number.	Height in inches.	Weight in pounds.	Body-surface area in square metres.	Age in years.	Fasting blood-glucose values in mg./100 c.c.
34	65.0	115.0	1.50	24.21	82.65
35	60.5	108.0	1.44	22.14	82.99 82.99
36	64.0	98.0	1.43	22.17	85.41
37	60.5	104.0	1.42	19.71	81.30
38	63.0	116.0	1.52	21.97	75.47
Arithmetic mean.	61.53	103.72	1.427	20.845	82.006
Standard deviation.	±2.36	±13.61	±0.10	±2.22	±9.29

The regression equation for the above values connecting the fasting glucose values with the body-surface area and the ages between 17 and 25 years is given by the expression, $y = +45.443 + 15.456 x_1 + 0.696 x_2$, where y represents the blood-glucose values in mg. per cent, x_1 , the body-surface area in square metres and x_2 , the age in years.

DISCUSSION.

Fisher's (1944) statistical test shows that the coefficients 15.456 and 0.696 are not significant at the usual 5 per cent level, the 't' values being only 0.999 and 0.964 (instead of at least 2.00 for $n=35$ to be significant at 5 per cent level), thereby indicating that the individual variations are much more than can be accounted for by a regression equation of this type and that by chance alone the fasting blood-glucose values so calculated could be exceeded by the actual tests. This finding, therefore, stands in contrast with the result previously reported by Variyar *et al.* (*loc. cit.*) in the case of male subjects, viz. $y = -14.120 + 70.500 x_1 - 0.841 x_2$ in which the coefficient of x_1 was very highly significant.

It is a well-known fact that blood-glucose values are subject to hormonal control. It may naturally be inferred, therefore, that the variations are partly due to the different phases of the menstrual cycle and that unless this fact is also taken into account in fitting a regression equation the fasting blood-glucose values cannot be accurately predicted. This leads also to the conclusion that a single blood-sugar value may not be accurate in a young woman as in a young man. It is interesting to note here that almost the same observation (the unreliability of a single estimation) was made in 1930 by Rathery *et al.* (quoted by Gradwohl, 1938). The fasting blood glucose in relation to menstrual cycle will, therefore, be the subject of a subsequent communication.

SUMMARY.

Statistical analysis of fasting blood-glucose values of 38 normal women between the ages of 17 and 25 years shows that true fasting blood glucose of young females cannot be represented by a regression equation connecting body-surface area and age alone.

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VARIYAR, M. C., and MANNADI NAYAR, A. S. (1946) *Ind. Jour. Med. Res.*, **34**, p. 175.

UTILIZATION OF SOYA-MILK PROTEIN FOR THE FORMATION OF BLOOD PROTEINS.

BY

H. S. R. DESIKACHAR,

S. S. DE,

AND

V. SUBRAHMANYAN.

*(An Inquiry on the Nutritive Value of Soya-Bean Milk under the Indian
Research Fund Association.)*

*(From the Department of Biochemistry, Indian Institute of Science,
Bangalore.)*

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INTRODUCTION.

THE biological assay of proteins for their nutritive value is usually done by the growth and nitrogen-balance methods. The methods developed by Osborne, Mendel and Ferry (1919), Thomas (1909), Mitchell and Carman (1926), Mitchell, Burroughs and Beadles (1936) and Chick, Hutchinson and Jackson (1935) are being widely used by all workers in the field of protein nutrition. These methods measure the nitrogen economy of the experimental animal in the matter of utilizing the protein for maintaining the various functions of protein in metabolism. Although this method of assay may not give us an idea as to the value of the protein for satisfying the requirements of the individual metabolic processes, it gives us a fairly comprehensive picture of the total value of the protein for satisfying all the functions of dietary protein put together.

In recent years, however, certain new methods have been developed for assaying the nutritive value of proteins which are based on the capacity of dietary protein for maintaining certain individual vital functions of protein in metabolism. These include the capacity of protein for (i) hæmopoiesis, (ii) plasma-protein production and (iii) liver-protein regeneration (Damodaran and Vijaya Raghavan, 1943; Orten

and Orten, 1946; Seeley, 1943; Kosterlitz, 1944; Harrison and Long, 1945). While no substitute method can claim to be as satisfactory as the nitrogen-balance method for evaluating the total nutritive value of proteins, these later methods mentioned above, in conjunction with the nitrogen-balance method, will add useful additional information to the nutritive value of the proteins.

It was on this consideration that the present work on the nutritive value of soya-bean milk protein was taken up. Our previous work on the nutritive value of the proteins of soya-bean milk by the growth and nitrogen-balance methods has shown that the proteins of soya-bean milk are about 90 per cent as efficient as the proteins of cow's milk regarding their nutritive value (Dcsikachar, De and Subrahmanyam, 1946). This paper deals with the comparative value of soya-bean milk protein and casein for hæmopoiesis as well as its value for maintaining the serum-protein levels in rats.

Part I.

COMPARATIVE VALUES OF SOYA-BEAN MILK PROTEIN AND CASEIN FOR HÆMOPOIESIS.

The method used was the same as the one developed recently by Damodaran *et al.* (*loc. cit.*). It consists chiefly in producing artificial anæmia in young rats (80 g. to 90 g. in weight) by an intraperitoneal injection of phenyl hydrazine and measuring the capacity of the experimental protein for restoring the normal levels of hæmoglobin and red blood corpuscles. On the fourth day after the injection there is a maximum fall in the percentage of hæmoglobin and in the red cell count. After this period, there is a gradual increase in the hæmoglobin percentage and in the red cell count, both of which reach normal levels on the 11th or 12th day after the injection. The percentage increase in the hæmoglobin and r.b.c. levels over those in the anæmic state gives a measure of the value of the protein for hæmopoiesis.

The soya-bean milk protein for the experiment was prepared by precipitation after adjusting the acidity to pH 4.2. The coagulum was collected after centrifuging, washed with water and dried on sheets of filter-paper in a current of warm air at about 45°C. The casein-like preparation thus obtained was finally powdered and used for the experiment.

The soya-bean milk protein was fed to the rats at 5 per cent level. Casein fed at the same level served as a control for comparison. Six rats were used in each group receiving the milk proteins. The composition of the diets was as follows:—

Starch	71 per cent.
Cane sugar	10 "
Fat (coco-nut oil)	10 "
Protein	5 "
Salt mixture (Weeson's, 1932)	4 "

A preparation of yeast extract was given to serve as the source of vitamins of the B complex. Sufficient amounts of vitamin A and calciferol were also

administered. Apart from the salt mixture, a solution of a mineral mixture containing iron, copper and manganese in optimum concentrations was also given.

The data bearing on the hæmoglobin and erythrocyte contents of the blood of the rats on the 4th and 12th days after the injection of phenyl hydrazine are given in Table I:—

TABLE I.
GROUP 1: 5 per cent casein diet.

Rat number and sex.	HÆMOGLOBIN IN G. PER 100 C.C. BLOOD.			R.B.C. IN MILLIONS PER C.M.M. BLOOD.		
	4th day.	12th day.	Percentage increase.	4th day.	12th day.	Percentage increase.
1	8.5	11.5	35.3	3.58	5.07	41.6
2	8.5	11.2	31.8	3.72	5.11	37.4
3	8.2	11.5	40.3	3.81	5.22	37.0
4	8.5	11.2	31.8	3.60	5.69	58.1
5	8.2	11.0	34.1	3.66	5.80	58.5
6	8.5	12.2	43.6	3.41	4.91	46.3
Average ...	8.4	11.5	36.2	3.63	5.30	45.5

GROUP 2: 5 per cent soya-bean milk protein diet.

1	8.5	11.5	35.3	3.40	5.47	40.3
2	9.0	12.0	33.3	3.72	5.26	41.4
3	8.5	11.8	38.8	3.67	5.27	43.6
4	8.5	11.2	31.2	3.50	5.02	49.1
5	9.2	12.2	32.6	4.08	5.87	43.8
6	8.8	11.5	30.7	3.92	5.93	51.2
Average ...	8.8	11.7	33.5	3.80	5.47	44.9

These results show that soya-bean milk protein is only slightly less efficient than casein for the regeneration of hæmoglobin and red blood cells. These observations are in conformity with those of our earlier findings by the growth and nitrogen-balance methods.

Part II.

SERUM-PROTEIN FRACTIONATION OF THE BLOOD OF RATS
RECEIVING SOYA-MILK PROTEIN.

It is now an established fact that deficiency of essential amino acids in the dietary protein prevents the maintenance of normal levels of plasma proteins in the blood. Albanese, Holt, Kajdi and Frankston (1943), while studying the effect of tryptophane deficiency on blood formation, observed that deficiency of tryptophane brings about a lowering of the plasma-protein level of the blood, the lower plasma-protein values becoming normal after supplementation with tryptophane. Seeley (*loc. cit.*) and Allison, Seeley, Brown and Anderson (1946) have developed a method of evaluation of proteins in hypoproteinæmic dogs by measuring the capacity of the protein for regeneration of the proteins of the plasma.

It was considered desirable, therefore, to study the serum-protein levels of the blood of rats receiving soya-bean milk protein against a control set receiving casein at the same level. The fractionation of the serum proteins was also carried out to obtain the ratio of the albumin and globulin in the blood as this ratio would be an index to the normal health of the rats.

Two groups of rats (six in each) about 60 g. in body-weight were kept on the casein and soya-bean milk protein diets for two months, the diet being fed *ad libitum*. The protein levels in both the diets were kept exactly at 10 per cent, the composition of the diet being: starch 66 per cent, cane sugar 10 per cent, coco-nut oil 10 per cent, salt mixture 4 per cent and protein 10 per cent. At the end of this period the rats were anaesthetized with ether and about 3 c.c. of blood were removed from each rat by heart-puncture. The serum samples were prepared and analysed for (i) total nitrogen, (ii) non-protein nitrogen and (iii) albumin nitrogen. Globulin nitrogen was calculated by difference. Nitrogen estimations were made by the micro-Kjeldahl method on 0.2 c.c. of plasma according to the methods of Albanese *et al.* (*loc. cit.*). In the albumin determinations, the usual precautions as suggested by Robinson, Price and Hogden (1937) were followed.

The results on the serum-protein fractionation of the blood of individual rats receiving the two proteins are given in Table II:—

TABLE II.
Casein group.

Rat number and sex.	Total nitrogen in mg. per 100 c.c. serum.	Non-protein nitrogen in mg. per 100 c.c. serum.	Protein nitrogen in mg. per 100 c.c. serum.	Albumin nitrogen in mg. per 100 c.c. serum.	Globulin nitrogen in mg. per 100 c.c. serum.	Albumin : globulin.
1	985	50.2	934.8	662.1	272.7	2.42
2	921	46.8	874.2	618.4	255.8	2.41
3	1.041	56.6	984.4	696.7	287.8	2.42
4	962	49.2	912.8	650.6	262.2	2.48
5	954	52.1	901.9	651.1	250.8	2.59
6	920	50.6	869.4	620.6	248.8	2.49
Average ...	964	50.9	913.1	649.9	263.2	2.47

TABLE II—concl'd.

Soya-bean milk protein group.

Rat number and sex.	Total nitrogen in mg. per 100 c.c. serum.	Non-protein nitrogen in mg. per 100 c.c. serum.	Protein nitrogen in mg. per 100 c.c. serum.	Albumin nitrogen in mg. per 100 c.c. serum.	Globulin nitrogen in mg. per 100 c.c. serum.	Albumin: globulin.
1	956	52.2	903.8	621.4	282.4	2.20
2	924	46.6	877.4	601.7	275.7	2.18
3	978	50.6	927.4	643.8	283.6	2.37
4	899	50.1	848.9	620.6	228.3	2.72
5	967	51.0	916.0	649.3	266.7	2.43
6	896	53.2	842.8	600.1	242.7	2.47
Average ...	937	50.6	886.1	622.8	263.2	2.39

The results tabulated above indicate that on the average both the total protein percentage as well as the albumin percentage are slightly higher in the casein-fed group than in the group receiving the soya-bean milk protein. Taking 6.25 as the conversion factor, the total protein and albumin figures are 5.71 per cent and 4.06 per cent in the casein group, while the corresponding figures in the other group are 5.54 per cent and 3.89 per cent. The differences are, however, very slight. The albumin: globulin ratios are also nearly the same in both the groups, showing that the rats on the soya-bean protein diet maintained normal health.

SUMMARY.

1. The protein of soya-bean milk was found to be about 90 per cent as efficient as casein for hæmopoiesis in the rat.
2. The protein of soya-bean milk could maintain normal albumin and globulin levels in the blood of rats receiving it as their sole source of protein. The albumin: globulin ratio is also normal.
3. As casein is the chief protein of cow's milk, these results bear out in a general way the previous findings by the nitrogen-balance and growth methods which showed that soya-milk protein is about 90 per cent as efficient as cow's milk protein in its nutritive value.

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PROTEIN VALUE OF SOYA-BEAN MILK: HUMAN FEEDING EXPERIMENTS.

BY

H. S. R. DESIKACHAR,

S. S. DE,

AND

V. SUBRAHMANYAN.

*(An Inquiry on the Nutritive Value of Soya-Bean Milk under the Indian
Research Fund Association.)*

*(From the Department of Biochemistry, Indian Institute of Science,
Bangalore.)*

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PREVIOUS experiments on rats carried out by us on the nutritive value of soya-bean milk protein showed that its biological value was about 90 per cent of that of cow's milk proteins (Desikachar, De and Subrahmanyam, 1946). The results of experiments on human subjects to study the protein utilization from soya-bean milk compared with cow's milk are presented in this paper.

The paper deals with the comparative utilization of the proteins of cow's milk and soya-bean milk curds fed along with the South Indian rice diet. The curd proteins have been used for comparison rather than the milk proteins because in poor South Indian families milk is generally used after souring it.

COMPARATIVE ECONOMY IN THE UTILIZATION OF THE PROTEINS OF SOYA-BEAN AND COW'S MILK CURDS.

Preparation, food intake, etc.—The South Indian diet chosen for the experiment was similar to the one used by most rice-eaters belonging to poor families. It consisted of cooked rice, a pulse soup (*Sambar*) and vegetables such as brinjal and amaranthus. Each experimental subject received daily as a basal diet about 1½ lb. rice, 40 g. tur dhal (*Cajanus indicus*), 10 g. of black gram (*Phaseolus mungo*) and Bengal gram (*Cicer arietinum*), 1 oz. vegetable oil (ground-nut), small amounts

of spices and condiments along with 100 g. of brinjal and amaranthus. One lb. of cow's milk curd or soya-bean milk curd was given along with the basal diet. The average caloric intake of food by a typical subject was as follows :—

	Calories.
Rice, tur dhal, black gram and Bengal gram, and vegetables	2,700
Milk solids apart from fat	160
Fat (vegetable oil and fat contributed by milk)	400
Total	3,260

The average distribution of protein in the basal diet was as follows :—

Source of protein in food.	Average intake of protein per day in g.
Rice	46·8
Tur dhal	7·8
Bengal gram	1·5
Black gram	1·1
Spices, condiments, tamarind, etc.	1·2
Brinjal	1·3
Amaranthus	2·9
Total	62·6

The addition of 1 lb. of milk along with the above basal diet would contribute about 10 g. to the protein intake forming about 1/7 of the total protein intake. Although the inclusion of 1 lb. of cow's milk is a rather liberal quantity compared with the actual intake of cow's milk in a poor family, it was nevertheless felt that the intake of 1 lb. of soya-bean milk per head per day is a quantity which a poor family can afford to take. Hence, it was decided to give about 1 lb. of milk per head per day.

The soya-bean milk samples were prepared daily and curdled for use. Fresh cow's milk got from a local dairy was used as a source of cow's milk curd. The milk samples were analysed daily for nitrogen.

The food was prepared by a trained cook and weighed amounts were served to the subjects. *Uppumav*, a rice preparation common in South India, was served during breakfast and tea-time while cooked rice along with *Sambar*, vegetable curry and curd were served during lunch and dinner.

Experimental.—The general plan of the experiment was to study the protein utilization by six healthy human subjects receiving a poor rice diet. There were three experimental periods each of one-week duration, five days being allowed as a rest period between two consecutive experimental periods. During the first period cow's milk curd was given along with the basal diet, while during the second period an equivalent quantity of soya-bean milk curd was given. The last period served as a control period when only the basal diet was served.

The total caloric intake as well as the protein intake by each subject was carefully controlled and restricted. During each of the three periods the total caloric intake by each subject was the same. The protein intake contributed by the basal food as well as the milk was also the same during the first two periods. The total caloric intake to be given to each subject during the three periods was determined by a separate feeding trial for one week in a preliminary period. The average intake per day during this period was maintained during the experimental periods. In the last experimental period when no curd was given, the caloric contribution made by curd in the previous experiments was made up by the calculated quantity of fat.

The last four days during each experimental period served as the collection period for urine and faeces. The urine samples were made up to volume on each day, an aliquot portion being preserved for analysis. The faecal samples for each subject were pooled together, the nitrogen analysis being made at the end of the experiment. The data for individual subjects regarding the total caloric and protein intake, as well as average daily nitrogen excretion in the urine and the faeces are presented in the Table:—

TABLE.

Subject.			Caloric intake per day.	Protein intake 'in g. Basal + Milk.	Urinary excre- tion per day (nitrogen in g.).	Faecal excretion per day (nitrogen in g.).
PERIOD 1:						
K. B.	3,000	58.5 + 10.35	3.344	3.412
M. N.	2,800	54.5 + 10.18	3.112	3.106
M. V.	2,800	54.5 + 10.18	2.986	3.165
S. M.	3,000	58.5 + 10.35	3.421	3.125
K. S.	2,800	54.5 + 10.18	3.081	3.169
S. O.	2,600	51.0 + 9.72	2.952	2.862
PERIOD 2:						
K. B.	3,000	58.5 + 10.35	3.496	3.465
M. N.	2,800	54.5 + 10.18	3.285	3.123
M. V.	2,800	54.5 + 10.18	3.132	3.112
S. M.	3,000	58.5 + 10.35	3.578	3.026
K. S.	2,800	54.5 + 10.18	3.151	3.014
S. O.	2,600	51.0 + 9.72	3.012	2.921
PERIOD 3:						
K. B.	3,000	58.5	2.946	3.287
M. N.	2,800	54.5	2.816	3.120
M. V.	2,800	54.5	2.258	3.116
S. M.	3,000	58.5	3.012	3.102
K. S.	2,800	54.5	2.721	3.289
S. O.*

* For unavoidable reasons this subject had to be excluded at the end of the second experimental period.

The results of some of the experiments are shown in Table I :—

TABLE I.

Antigenic potency of different batches of antirabic vaccine prepared at the Central Research Institute, Kasauli.

Batch number.	Dilutions of the test dose injected.								Controls.				Protection against number of m.l.d.
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	
3,436	...	*0/1	0/4	0/5	0/6	0/4	1/5	4/5	3/6	3/6	1,000,000†
2,060/9/1	1/6	1/6	1/6	>10,000
2,666/7/8	1/6	1/6	0/6	1/4	3/4	0/6	>10,000
2,775/6	1/6	0/5	1/5	>10,000
2,987/8	2/5	0/5	0/5	10,000
3,112/3	2/5	3/6	1/5	1/5	>10,000
3,296/7	2/3	2/6	3/6	0/4	2/4	4/4	1/5	10,000
3,436	2/6	2/6	1/5	0/4	1,000,000
3,096/7	...	0/5	0/6	0/6	0/6	>10,000
3,090/1	...	3/5	0/3	0/4	0/3	4/5	3/5	1/5	0/6	1,000
3,085/6	...	3/6	0/6	1/5	0/4	1,000
3,114/5	...	1/3	0/5	1/5	0/6	10,000

* Number of deaths.
Number of mice injected.

† Approximate figures.

TABLE I—concl'd.

Batch number.	Dilutions of the test dose injected.								Controls.				Protection against number of m.l.d.
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	
2,666/7/8	2/6	1/5	3/6	0/5	2/5	2/5	1/5	10,000
2,638/9	1/6	2/6	0/6	2/6	10,000
3,296/7	2/5	1/5	2/6	1/6	10,000
3,299/0	3/5	2/5	2/6	1/5	2/5	2/5	1/5	1,000
3,797	...	4/6	1/6	1/4	1/4	1,000
3,796	...	6/6	3/6	4/6	1/3	6/6	1/5	1/4	<100
3,795	...	3/5	5/6	4/6	0/6	<100
3,794	...	3/6	4/6	3/6	0/6	100

The various batches of antirabic vaccine prepared by the same technical staff varied markedly in antigenicity, some batches conferred protection against 100 m.l.d. only, others protected mice against even one million m.l.d. of the fixed virus. Dunlap (1943) found that there were considerable variations in the titre of brain in different sheep, viz. 10⁻⁵ to 10⁻⁷. Veeraraghavan (1946) confirmed these findings. It is, therefore, not surprising that Semple's vaccines display such a marked diversity in their antigenic potencies.

It is possible that the antigenicity is markedly affected by the high concentration of the antiseptic acting on the rabies virus for a fairly long time. The lethal action of various antiseptics on the rabies virus was re-investigated with a view to determining the minimal amount of antiseptic which should be added to rabies brain for the preparation of the vaccine; a 5 per cent suspension was generally used. The summary of the results is as follows:—

- 0.2 per cent carbolic acid killed the virus in 29 to 36 days at room temperature.
- 0.2 " " did not kill the virus in 13 days at 37°C.
- 0.5 " " killed the virus in 25 days at room temperature.
- 0.5 " " did not kill the virus in 15 days at room temperature.
- 0.5 " " killed the virus in 3 days at 37°C.
- 1.0 " " in 8 per cent vaccine killed the virus in 12 hours at 37°C.
- 0.4 per cent formalin killed the virus in 15 days at room temperature.
- 0.5 per cent chloroform did not kill the virus in 15 days at room temperature.
- 0.5 " " killed the virus in 25 days at room temperature.

The antigenicity of different brews of vaccine treated with different concentrations of the antiseptic was also determined. The results are presented in Table II.

It will be seen from the data presented in Table II that (a) the antigenicity of different brews of rabid brain is not affected to a lesser extent by formalin and chloroform than by carbolic acid under the conditions of the experiment and (b) the different brews vary markedly in antigenicity.

TABLE II.
Antigenic potency of different experimental brews of antirabic vaccines.

Batch number.	Dilutions of the test dose injected.								Controls.			Protection against number of m.l.d.
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	
0.2 per cent carbolic, 42 days at room temp.	1/5	1/5	0/5	0/5	0/5	3/5	3/6	0/5	>100,000
0.1 per cent formalin, 42 days at room temp. ...	2/2	5/6	2/3	2/5	0/5	0/6	1,000
Formalin 0.1 per cent, 9 days at room temp.	3/6	3/6	0/6	0/5	0/3	10
Carbolic 0.2 per cent, 9 days at room temp.	0/2	1/6	0/6	0/5	0/3	4/6	1/5	0/5	>100
Carbolic 0.5 per cent, 15 days at room temp.	5/6	2/6	2/6	3/6	0/5	5/6	3/6	3/5	1,000
Chloroform 0.5 per cent, 15 days at room temp.	4/4	3/5	4/6	1/6	2/6	2/5	1,000
Carbolic 0.2 per cent, 10 days at 37°C.	...	1/5	0/5	0/3	0/5	0/3	0/3	...	4/5	3/6	3/6	1,000,000
Carbolic 0.2 per cent, 13 days at 37°C.	2/4	3/5	2/5	0/5	2/4	4/4	1/5	10,000

The effect of a lower concentration of carbolic acid than that normally used was determined in a further experiment. The results were as follows :—

	10^{-5}	10^{-4}	10^{-3}	10^{-2}
0.5 per cent carbolic, 3 days at 37°C.	1/6	0/6	4/6	4/5
1.0 per cent carbolic, 1 day at 37°C.	0/5	0/6	4/6	4/6

It is evident that a reduction in the concentration of carbolic acid in the preparation of the vaccine did not improve the immunizing power of the killed vaccine. There doesn't seem any point in making Semple's vaccine by adding 1 per cent carbolic acid in the beginning and diluting it after incubation. It would be simpler to add 0.5 per cent carbolic acid to 5 per cent vaccine and incubate for 3 days at 37°C.

It is not practicable to test the antigenicity of each different brew of antirabic vaccine *in vivo*, as it would involve the use of a very large number of animals and considerable time would also be required to arrive at a definite conclusion. Attempts were, therefore, made to devise a suitable *in vitro* test for the standardization of vaccines.

In vitro titration.—Havens and Mayfield (1932) and Greval (1932) reported positive complement-fixation tests in rabies. The following technique was employed in the preparation of antirabic sera :—

Rabbits were immunized by the intraperitoneal route with the carbolyzed antirabic vaccine prepared at the Central Research Institute, Kasauli. The vaccine was first administered every day, with the increase in dosage from 0.2 c.c. to 4.0 c.c., the interval between the injections was gradually increased to 4 days. The rabbits were bled 5 weeks after the commencement of immunization, the sera (batch I) proved to be poor in complement-fixing antibodies.

In the next series of experiments, an experimental brew of antirabic vaccine (5 per cent vaccine in distilled water containing 1 per cent chloroform and left at room temperature for three weeks) was used in the immunization of rabbits. Intraperitoneal injections were given at 4-day intervals, the dose was increased from 2.5 c.c. to 4.0 c.c., these animals were bled six weeks after receiving the first dose of the vaccine, the sera (batch II) had a high complement-fixing antibody content.

The complement-fixation test was carried out as follows : A two-month-old sample of antirabic vaccine under test was centrifuged at moderate speed for 15 minutes, the supernatant fluid was heated at 56°C. for 30 minutes and an appropriate dilution was selected for use after preliminary trials. The immune serum was also inactivated at 56°C. for 30 minutes. The m.h.d. of guinea-pig serum kept overnight in the refrigerator was determined and appropriate amounts of the m.h.d. of the complement were used in the test. For the titration of antirabic serum, 0.5 c.c. of a 1/8 dilution of the antigen was added to 0.25 c.c. of double dilutions of antirabic serum, 2 m.h.d. of complement were added and the mixtures were incubated at 37°C. for 90 minutes, sensitized cells were added in 0.5 c.c. amounts and the tubes were returned to the incubator for another hour. A two-month-old normal brain suspension was used as a control. The results are presented in Table III.

It will be observed from Table III that specific complement-fixing antibodies are demonstrable in antirabic serum (batch II).

The antirabic serum (batch II) was consequently used in the *in vitro* titration of different brews of antirabic vaccines. The results are shown in Table IV:—

TABLE IV.

Complement-fixation test with different brews of antirabic vaccine.

Antirabic serum 1/100–0.5 c.c.; antigen 1/10–1/30–0.1 c.c. to 1.0 c.c.; saline 0.9 to 0.0 c.c.; 2 m.h.d. complement = 90 minutes 37°C.; sensitized cells 0.5 c.c.—1 hr. 37°C.

Batch number.	<i>In-vivo</i> titre.	Varying amounts of antigen.									
		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
2,060/9/1	>10,000	CL	ACL	ACL	Tr	NL
2,666/7/8	>10,000	CL	ACL	Tr	NL
2,638/9	10,000	CL	CL	CL	ACL	PL	Tr	NL
2,775/6	>10,000	CL	CL	ACL	ACL	ACL	Tr	NL
2,987/8	10,000	CL	CL	CL	CL	ACL	ACL	ACL	NL
3,436	1,000,000	CL	Tr
3,296/7	10,000	CL	ACL	Tr
3,096/7	>10,000	CL	CL	CL	Tr	...
3,090/1	1,000	CL	ACL	Tr
3,085/6	1,000	CL	PL	Tr
3,114/5	10,000	CL	CL	CL	CL	CL	CL	ACL	ACL	ACL	PL

Antigen diluted 1/20.

2,666/7/8	>10,000	CL	ACL	PL	Tr
2,638/6	10,000	CL	CL	PL	Tr
3,296/7	10,000	CL	ACL	Tr
3,296/0	1,000	CL	ACL	Tr

Antigen diluted 1/10.

3,112/3	>10,000	CL	CL	CL	CL	Tr
41 (0.5 per cent carbolic).	>100	CL	CL	CL	ACL	PL	Tr
43 (0.4 per cent formalin).	100	CL	ACL	ACL
3,436	1,000,000	CL	CL	ACL	ACL	ACL	PL	PL	Tr

It will be evident from Table IV that there is no strict correlationship between the antigenic potency of the vaccines as tested *in vivo* and their complement-fixing power.

Havens and Mayfield (*loc. cit.*) reported that specific flocculation of rabies virus occurs in appropriate dilution of immune rabbit and guinea-pig serum. Experiments were consequently put up to devise a suitable flocculation test for the standardization of antirabic vaccines.

Flocculation test.—The supernatant fluid obtained from the antirabic vaccine was mixed in 0.5-c.c. amounts with an equal volume of double dilutions of antirabic serum. The mixtures were incubated at 45°C. for at least 23 hours, and the results are shown in Table V:—

TABLE V.

Flocculation test with different brews of antirabic vaccine.

Antigen undiluted : Supernatant 0.5 c.c. ; antirabic serum 0.5 c.c. —1/8 to 1/128 dilution.

Batch number.	<i>In vivo</i> potency.	Antirabic serum dilutions.				
		1/8	1/16	1/32	1/64	1/128
2,666/7/8	>10,000	ff	ff	f	...	23 hrs.
2,638/9	10,000	fff	ff	f	...	3 hrs.
		fff	fff	ff	f	23 hrs.
3,296/7	10,000	fff	ff	f	...	3 hrs.
		fff	fff	ff	f	23 hrs.
3,299/0	1,000	fff	ff	f	...	3 hrs.
		fff	fff	fff	...	23 hrs.
Normal brain	fff	fff	f	...	23 hrs.

It will be noticed from Table V that flocculation test cannot be used as a guide for determination of the exact antigenic potency of these vaccines.

In view of the non-specific flocculation reaction observed by the use of normal brain suspension, an attempt was made to absorb the non-specific antibodies from the antirabic serum. The absorbed serum unfortunately failed to give a flocculation reaction with rabid brain extract.

SUMMARY AND CONCLUSIONS.

1. An investigation into the antigenicity of different batches of antirabic vaccine prepared at the Central Research Institute, Kasauli, revealed wide variations in their potency. The vaccines were tested *in vivo* in mice by the modified Habel's technique.

2. The complement-fixation and flocculation tests could not be used as screen tests for the titration of antirabic vaccines.

In view of the considerable variations in the potency of different batches of Semple's antirabic vaccines, it would be advisable to pool a fairly large number of vaccines to arrive at a more or less uniform titre.

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STUDIES ON TYPHUS IN THE SIMLA HILLS.

Part IX.

ON THE LIFE-HISTORY OF *TROMBICULA DELIENSIS*, WALCH, A SUSPECTED VECTOR OF TYPHUS IN THE SIMLA HILLS.

BY

DEV RAJ MEHTA, M.Sc., Ph.D. (Cantab.),
*Entomologist, Typhus Inquiry under the Indian Research Fund
Association.*

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A STUDY of the life-history of *Trombicula deliensis*, Walch, is of more than just academic interest because the larval forms of this mite are known to attack man possibly producing pathological results. Walch (1922-23) has studied the possibility of typhus transmission through the agency of this mite. His suspicions were mainly founded on the geographical distribution of *T. deliensis*, some proof of the transmission and the evidence that it belongs to Nagayo's (Nagayo *et al.*, 1921) tsutsugamushi group of mites. Within that group, it resembles very closely the larva of *Trombicula akamushi*, an accepted vector of Japanese River Fever. Judging from the larval characters of *T. deliensis* and *T. akamushi*, Gater (1930) showed that *T. deliensis* is merely a form of the same species, namely *T. akamushi*. Similarly, considering that tsutsugamushi disease is habitually associated with *T. akamushi*, Brumpt, or *T. deliensis*, Heaslip (1941) pointed out that the latter is at most a variant of *T. akamushi*.

In the Simla Hills, the seasonal distribution of the five species of *Trombicula* found on rats has been recorded in an earlier communication (Mehta, 1937). It was then emphasized that larvæ of *T. deliensis* were particularly abundant during the time when cases of typhus XK occurred at Kasauli and Sabathu in the Simla Hills. Although no definite evidence incriminating this mite in the transmission of typhus of the XK serological type is available, there have been instances in the Simla Hills and elsewhere in which persons attacked by the larvæ of this mite developed typhus XK.

larvæ were recovered from the soil by lavage method. Tragardh's illumination method did not produce any larvæ from similar situations although other genera of mites were found.

The larvæ remained attached to the host for a fairly long time varying from a fortnight to a month or more depending on temperature. It was ascertained that at temperatures ranging between 75°F. and 80°F. the larvæ remained on the host for 15 days and later dropped off after repletion. Subsequently, with the advent of cold weather this period was gradually prolonged. During severe cold weather conditions, when the temperature was lowered to 32° to 55°F., these larval mites either dropped off at an early stage of attachment or remained on the host for about two months.

THE NYMPH.

For raising nymphs in the laboratory the method adopted has already been described. The engorged larvæ move about in the culture jar for some time before passing into a quiescent stage, viz. the nymphochrysalis (Henking, 1882, quoted by Banks, 1915). The nymphs hatched out in 15 days at temperature varying from 23° to 25°C. (73.4° to 77°F.).

Following Nagayo's technique (Nagayo *et al.*, 1917) it has been suggested by Walch (1923) that the larvæ must be at least 300 microns in length to be able to metamorphose into the nymphal stage. In the present studies this has been confirmed but the nymphs which developed from such larvæ did not live long and were very weak. It is, therefore, essential that larvæ must be fully engorged in order to complete their life-cycle successfully.

A freshly-hatched nymph measures 400×272 microns and is easily distinguished from the larva by the presence of eight legs. The shape of the body is also different, being markedly divided into a small anterior and a larger posterior part by a deep constriction, thus forming a figure of eight. The mouth parts are adapted for a vegetarian mode of existence and the eyes are indistinct.

For the description of the nymph of *Trombicula deliensis* Oudemans' (1909) nomenclature as reproduced by Walch (1923) has been followed.

The gnathosoma denote the mouth parts which include the chelicerae and the palps. The latter are five-jointed for which the terms trochanter, femur, genu, tibia and tarsus have been employed. The tarsus or appendiculum ('thumb') (Nagayo *et al.*, 1917) is about as long as the palpal claw and it is not club-shaped. The bases of the palps lie together forming a plate which represents the hypostome. The claw is single. The palps are covered with sparsely-scattered fine pores. A minute seta is present on the appendiculum and a larger one on the article behind. About eight small bristles are grouped together around the tip of the appendiculum. The palpal spurs on the inside of the tibia near the base of the claw described by Walch (1923) are not clearly seen. The claw and the appendiculum are 20 and 30 microns long respectively. These measurements correspond to those of the nymph of *Trombicula akamushi* described by Nagayo *et al.* (1917).

The chelicerae (mandibulæ) (Walch, 1923) or the mandibles (Nagayo *et al.*, 1917) are 132 microns in length and provided with a sharp, claw-like process or hook at

the anterior end which is 40.8 microns long. It is finely serrated at the concave edge. There is an ill-defined membranous prominence ('Digitus fixus') near the base of the hook which is also termed the 'Upper chela'. The epistome, which is a prolongation of the dorsum of the body, covers the mandibles at their bases and bears a feathered hair 23 microns in length.

The propodosoma include the parts of the body where the first and second pairs of legs are situated. Besides the organs of locomotion the scutum and the crista metopica form the important structures in this division. The latter is a tube-like fold of chitin which extends anteriorly from the scutum towards the gnathosoma. It is hairless throughout. Posteriorly it surrounds the areola sensilifera (scutum). The scutum bears a pair of sensory hairs (pili sensoriales) (Walch, 1923) arising from pits. The sensory hairs are long and slender measuring 109 microns with an almost naked proximal and a feathered distal half. The distance between pits is 27 microns. The general appearance of the scutum, according to Walch (1923), is very similar to that of *Trombicula akamushi*; and he has laid special stress on the presence of two posterior lobes situated behind the pits which are clearly seen in the Indian species on fine focusing. The length of the sensory hairs and the distance between the pits in the nymph of *Trombicula akamushi* as given by Nagayo *et al.* (1917) are 100 and 20 microns respectively, showing a striking resemblance between *T. deliensis* and *T. akamushi*. These measurements of the Malayan species, as given by Walch (1923), vary slightly though his conclusions in this respect are the same.

The eyes appear to be indistinct in the nymph of *Trombicula deliensis*.

The metapodosoma include the parts of the body involving the third and fourth pairs of legs. The remaining part of the body is termed opisthosoma by Oudemans (*loc. cit.*).

The four pairs of legs are of varying length in the nymph. These are formed of six articles or joints. The first pair of legs is the longest, whilst the fourth, second and third pairs come next in the descending order. According to Berlese (1912), the relative length of the tibia and the tarsus of the first pair of legs and the relation of the length and breadth of the tarsus of this pair are of importance in distinguishing the different species. These measurements have, therefore, been taken of the Indian species and given below:—

The coxæ of the first and second pairs of legs lie close to each other and so do the coxæ of the third and fourth pairs. The distance between the anterior and posterior groups of the paired legs is 68 microns. The transverse distances between the pairs of the anterior group and pairs of the posterior group are 30.6 and 54.4 microns respectively. These measurements are nearly the same as given by Nagayo *et al.* (1917) for the nymph of *Trombicula akamushi*. The measurements of the various joints and the coxæ of the legs are given in Table I.

The relationship between the length and breadth of the tibia and the tarsus (joints V and VI) of the first pair of legs is 1 : 1 : 6 in the case of *Trombicula deliensis* as compared with 1 : 1 : 4 figured out by Nagayo *et al.* (1917) for the nymph of *Trombicula akamushi*.

TABLE I.

Measurements of legs of T. deliensis nymph.

Joints.	Measurements.	JOINTS.						CLAW.
		I.	II.	III.	IV.	V.	VI.	Microns.
		Microns.						
First pair	Length	23·8	61·0	47·6	54·4	68·0	108·8	13·6
	Breadth	23·8	32·3	34·6	37·4	39·1	40·8	
Second pair	Length	37·0	44·2	30·6	30·6	34·0	64·6	17·0
	Breadth	34·0	27·2	30·6	25·5	25·5	20·4	
Third pair	Length	39·0	40·8	30·6	27·2	35·7	61·2	15·3
	Breadth	34·0	27·2	27·2	23·8	23·8	18·7	
Fourth pair	Length	51·0	44·2	35·7	37·4	39·0	69·7	17·0
	Breadth	32·0	28·9	28·9	27·2	25·5	23·8	

Measurements of coxae (epimeres, Nagayo et al., 1917).

Measurements.	LEGS (PAIRS).			
	I.	II.	III.	IV.
Microns.				
Length ...	72.0	59.0	54.5	59.5
Breadth ...	35.7	34.0	41.0	37.4

Each tarsus is provided with two claws of varying lengths. Their measurements have already been given.

The abdomen is profusely clad with hairs of varying length which are characteristically feathered and are particularly larger and thicker towards the posterior part. In addition to the paired sensory hairs which have been described above the other hairs are almost evenly distributed. Their length varies from 22 to 44 microns and are pinnate. The hairs on the legs are small (22 microns) and pointed and are more numerous on the first pair.

The tracheal and stigmatal plates of a chitinous nature are not traceable in the nymph.

The genital opening is situated in the median line ventrally on the abdomen behind the coxæ of the fourth pair of legs and is surrounded by a pair of flat oval plates known as the sucking discs. There are three small hairs about 17 microns in length on either side of the genital opening. They differ from the other body hairs in not being conspicuously feathered. Sexual differentiation is not evident. The length and breadth of the genital opening are 56 and 51 microns respectively.

The anal opening is situated behind the genital opening and is 40 microns in length.

HABITS OF THE NYMPH.

The precise details of the habits of the nymphs of *Trombicula*, particularly those which belong to the *tsutsugamushi* group of mites, remained a mystery for a very long time. Miyajima and Okumura (*loc. cit.*) were the first to describe the life-cycle of *Trombicula akamushi* and showed after a number of trials that the most suitable food for the nymphs is the juice of potatoes, melons and other vegetables, but they seem to dislike sour fruits such as apples and oranges. These observations were, however, not confirmed by later workers. In fact, Hatori (*loc. cit.*), in his studies on the endemic *tsutsugamushi* disease of Formosa, failed in his attempts to develop the nymph of *Trombicula akamushi* on vegetable matter. Walch (1923) stated that the nymphs of *Trombicula* lived on decayed leaves, juices of plants such as the reed *Imperata*, *Artemesia vulgaris*, etc. In view of the conflicting opinions regarding the food of trombiculid nymphs, a study of their feeding habits was undertaken. Experiments were conducted to find out whether the nymphs of *T. deliensis* fed on juices of vegetables and sweet fruits. The following are the details of these experiments:—

During the summer of 1938, nymphs of *Trombicula deliensis* were kept in small glass-jars which contained moist, sterilized earth and were given sliced potatoes, carrots, melons and sweet apples in separate lots. In this manner 350 nymphs were tested. The slices of vegetables and fruits were replaced daily after the old ones had dried up. It was found that the nymphs did not feed on any of the food-stuffs mentioned above, and signs of starvation were apparent in about three weeks when the nymphs became sluggish and ultimately died within a month. There was a marked shrinking of the abdomen before they perished. Similar attempts to feed these nymphs on dried and pulverized rat's blood and faeces of rats also failed.

After several preliminary trials it was found that the best method to raise adults of *Trombicula deliensis* from nymphs and subsequently obtaining larvæ from the former consisted of keeping the nymphs in a glass-cage measuring 2'×1'×1' 6" as shown in the photograph (see Plate III). This cage was provided on the only open side with a door. On the metal floor of this cage about 3 inches thick sterilized moist earth was spread and small wild grass was grown in it to imitate natural field conditions. A small wire-netting cage (7"×4") containing a wild brown rat (*Rattus rattus*) was placed inside the glass-cage to attract any larvæ which might hatch out from the eggs laid by the adults. The earth inside the cage was kept sufficiently moist by daily sprinkling water during the summer months.

Sterilized oats and carrots were given as food to the rat daily. In this manner it was possible to raise adults of *T. deliensis* successfully from the nymphs. During the course of these experiments it was observed that the nymphs did not come on the surface of the soil to feed on the juices of plants grown in the glass-cage. When this soil was shaken nymphs which had apparently fed on decaying matter were observed. This was confirmed by crushing the nymph under a coverslip and subsequent examination under the microscope revealed a mass of dark decaying matter. Further experiments on the nymphs and adults of *T. deliensis* by keeping them alive on this material alone for a long period in small glass-bottles lent further support to this observation.

The nymphs, as a rule, are negatively phototropic and remain concealed under the soil at a depth of about two inches. This was easily seen by disturbing the soil when the nymphs came on the surface and after a short time went underneath again.

The growth of the nymph is marked by increase in size. A fully-fed nymph, which had lived for about six weeks, measured about 600 microns and before passing into the resting stage it measured about 900 microns. The metamorphosis from the nymph to the adult takes place underneath the soil when the former passes into a quiescent stage termed teliochrysalis by Miyajima and Okumura (*loc. cit.*). The adult mite hatched out after some time which could not be precisely determined as the latter remained hidden in the soil.

The nymphs which had hatched out from larvæ on the 11th July, 1938, developed into adults on the 19th September, 1938, thus taking about nine weeks including the quiescent period.

Like the nymphs the adult mites of *T. deliensis* were also found below the soil and did not come up to the surface unless the soil was disturbed. This aversion to light is probably responsible for the difficulty in the past to render a complete account of their life-history.

THE ADULT OR PROSOPON.

The adult or prosopon resembles the nymph very much and can be differentiated chiefly by its larger size and the presence of three pairs of sucking discs around the genital opening.

PLATE III.



Glass-cage for breeding of *T. deliensis*.

A freshly-hatched adult is of light orange colour and it appeared that possibly due to the advent of cold weather this pigment is very much intensified. It measured 864 microns long but after having lived for about a month it was found to be 1,000 microns long when it was fully fed. As in the nymph the body of the prosopon is divided into two unequal parts by a deep constriction situated between the coxæ of the third and fourth pairs of legs. The anterior part or the cephalothorax measures 513 microns at the widest points and the distance between the constricted part is 368 microns. The posterior portion, which is comparatively wider, measures 520 microns. There are individual variations in different specimens examined but the above figures give an idea of the measurements of a freshly-emerged adult.

The gnathosoma are represented by well-developed mandibles and maxillipalps. The latter are five-jointed including the basal joint and the general appearance is very much similar to that of the palp of the nymph. The thumb or the appendiculum is elongated and not club-shaped. It is 51 microns in length and 11.9 microns broad, and it is provided with several feathered hairs and a few short setæ. The claw is single and measures 20 microns. The third and fourth joints of the palp are provided with numerous bristles and feathered hairs but their distribution on the first and second articles is comparatively scanty.

The mandibles are prominent organs and chelate. The hook of the mandible measures 57.8 microns and is a thin blade.

In the propodosoma there are, as in the nymph, two pairs of legs, the scutum and the crista metopica which are distinct. The crista is a transparent chitinous tube which extends from the areola sensiligera (scutum) to the anterior part of the body towards the gnathosoma. It is about 112 microns in length. The scutum has a characteristic appearance and very much resembles that of the nymph. It is 51 microns wide and its greatest sagittal diameter is 30.6 microns. A pair of sensory hairs arise from pits on the scutum. In the specimen examined their length could not be accurately measured as they were broken at the distal end. The posterior chitinous lobes behind the pits are well marked and much more conspicuous than in the nymph. The distance between the sensory pits is 34 microns as measured from the roots of the sensory hairs. The eyes are not present which explains the negatively phototropic behaviour of the adult.

There are four pairs of legs, each with six joints excluding the coxæ or epimeres. These are thickly clad with feathered hairs and numerous bristles. The terminal joint ends in two claws. The first pair is the longest, then the fourth, the second and the third. The measurements of the individual joints of the legs, the coxæ and the claws have been given in Table II.

The other important organs on the abdomen for the critical systematic examination of the adult (prosopon) are the genital organs including the genital discs which are in three pairs in contrast to two pairs observed in the nymph. The genital opening lies in the mid-ventral line surrounded by the

TABLE II.

Measurements of legs of T. deliensis adult.

Joints.	Measurements.	JOINTS.						CLAW.
		I.	II.	III.	IV.	V.	VI.	Microns.
		Microns.						
First pair	Length ...	61.2	44.2	78.0	91.0	107.8	176.8	20.4
	Breadth ...	45.4	37.2	51.0	54.0	57.8	57.8	
Second pair	Length ...	44.2	40.8	44.2	54.0	61.0	91.8	23.8
	Breadth ...	44.0	35.7	37.4	37.4	34.4	33.0	
Third pair	Length ...	39.1	40.8	44.2	51.0	61.2	91.8	25.5
	Breadth ...	37.4	35.7	35.7	35.7	35.7	27.2	
Fourth pair	Length ...	47.6	54.4	57.8	61.2	78.2	115.6	24.4
	Breadth ...	40.8	34.0	35.7	35.7	35.7	30.6	

Measurements of coxae of adult T. deliensis.

Measurements.	LEGS (PAIRS).			
	I.	II.	III.	IV.
Microns.				
Length ...	119.0	74.8	105.4	105.4
Breadth ...	56.1	47.6	69.0	71.4

Microns.

Distance between the anterior and posterior groups of paired legs. 127.5

Transverse distance between the anterior group of legs. 54.4

Transverse distance between the posterior group of legs. 112.2

genital discs. The measurements of the genital opening and the sucking discs are as follows :—

			Microns.
<i>Genital openings :—</i>			
Length	139.4
Width	85.0
<i>Sucking discs :—</i>			
Length	23.8
Width	20.4

The anus is situated at a distance of 54.4 microns from the genital opening and it is 85 microns long and 74.8 microns wide.

The hairs on the abdomen are very thickly distributed and are of varying length. In the anterior part they are about 23.8 microns in length near the sides but towards the posterior portion they attain a length of about 47.6 microns. The latter type of hairs are feathered as in the other parts of the body but towards their distal extremity they are definitely knobbed. The hairs on the legs vary in length from 24 to 28 microns.

HABITS OF THE ADULT *T. deliensis*.

The adults can exist on decaying matter for a very long time. Under experimental conditions adults which had been kept in glass-bottles containing decaying matter on 19th September, 1938, remained alive beyond 20th March, 1939, and were quite healthy. No other kind of food was given to these mites.

It is claimed that the adults of *Trombicula deliensis* can tolerate extremely low temperatures. In Kasauli where these observations were made these mites survived during the winter when the temperature in the room was about 13°C. (55.4°F.) and sometimes even below this. It appeared, therefore, that this mite over-wintered in the adult stage. In this connection it may be mentioned that *Trombicula akamushi* also survived through the winter as a prosopon (Nagayo *et al.*, 1921).

Sexual dimorphism amongst these mites does not appear to occur. It has been shown by Miyajima and Okumura (*loc. cit.*) that the eggs of *Trombicula akamushi* are laid in the soil from which the larvæ hatch out subsequently. In the present study it has not been possible to isolate the eggs of *T. deliensis* from the experimental jars in which young larvæ of this mite had been recovered from time to time. From the adults which developed from nymphs in the laboratory during September 1938, larvæ were obtained on 10th October, 1938. From this it is evident that it took about a month for oviposition and incubation of eggs at the time when the temperature ranged about 25°C. (77.0°F.).

In all 10 larvæ were obtained from two adults of *Trombicula deliensis* and the time when these observations were made is recorded in Table III (p. 170).

TABLE III.

Emergence of larvæ of Trombicula deliensis.

Date adults first observed.	Date larvæ emerged.	Number of larvæ emerged.	Room temperature, °C.
19-9-1938	10-10-1938	3	25
19-9-1938	12-10-1938	1	...
19-9-1938	24-10-1938	1	...
19-9-1938	11-11-1938	1	...
19-9-1938	19-11-1938	2	14
19-9-1938	26-11-1938	1	...
19-9-1938	5-12-1938	1	...

From the above data it is evident that the larvæ of *T. deliensis* emerge in small numbers which suggests that the eggs are probably laid singly. If this be so, these observations confirm the observations of Miyajima and Okumura (*loc. cit.*) that the eggs of *Trombicula akamushi* are laid singly. There is further scope for more observations on the oviposition and hatching of eggs of *T. deliensis*.

SUMMARY AND CONCLUSIONS.

1. A detailed account of the life-history of *Trombicula deliensis* has been given including observations on the feeding habits of the Indian form of this mite and its immature stages.

2. A simple technique has been developed for the study of its life-history.

3. Details in regard to the morphology of the larva, nymph and the adult of *Trombicula deliensis* have also been recorded.

4. A study of the morphology and the habits of this mite suggest that *Trombicula deliensis* is so closely related to *Trombicula akamushi* that the former may justifiably be regarded as a variant of the latter.

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MODE OF ACTION OF RUSSELL'S VIPER (DABOIA) VENOM.

BY

M. L. AHUJA,

AND

A. G. BROOKS.

(From the Central Research Institute, Kasauli.)

[Received for publication, January 8, 1948.]

It is generally believed that Russell's viper (*daboia*) venom contains two active principles: (i) a 'fibrin ferment' capable of clotting blood, and (ii) an 'anti-fibrin ferment' which reduces the coagulability of blood. Other fractions—haemolysin, neurotoxin, leucolysin, etc.—are thought to play a minor part in bringing about the fatal issue following the bite of a Russell's viper.

The belief that *daboia* venom is both coagulant and anti-coagulant is based on the following well-known observations: When a lethal dose of the venom is injected intravenously death is almost instantaneous due to intravascular clotting. The clotting is generally visible to the naked eye, especially in the heart and larger blood vessels. This proves the presence of the 'fibrin ferment' in the venom. On the other hand, when venom is administered by the intramuscular route death takes place after some hours and the blood is rendered permanently incoagulable. The incoagulability was attributed by Lamb (1903), Calmette (1908) and Wall (1917) and other authorities to the supposed 'anti-fibrin ferment'.

The correctness of these observations is undoubted, their interpretation may, however, be questioned. On *a priori* grounds one is justified in doubting the possible existence of such antithetical fractions in the venom, for it is difficult to believe that the same substance will have diametrically opposed actions when given by the intramuscular and intravenous routes. The following observations are recorded to show that *daboia* venom acts principally, if not solely, as a powerful coagulant whether injected intravenously or intramuscularly and that no other fraction of importance exists in the venom:—

Histological examination of tissues of animals which died as the result of intramuscular injection of *daboia* venom showed that capillaries, particularly of the lung, liver and brain, were packed with blood clots and fibrin. So great was the deposition of fibrin in some tissues as to suggest that the blood had been rendered incoagulable because of de-fibrination. Accordingly, the following

experiment was carried out: The blood of a normal sheep was examined for fibrin content and was found to contain 350 mg. per 100 c.c. of plasma. This sheep was then given an intramuscular injection of 20 mg. of daboia venom. When the blood had reached the incoagulable stage it was again examined for fibrin content—no fibrin could be detected. The absence of fibrin from the sample examined did not of course exclude the possibility of an 'anti-fibrin ferment', for such a ferment, if present, could well account for the finding. That no such ferment was present in the incoagulable blood was proved as follows: To 1 c.c. of normal sheep blood was added 0.2 c.c. of the incoagulable de-fibrinated blood which had resulted from the injection of the venom. The clotting time of the normal sheep blood was reduced from 12 minutes to $2\frac{1}{2}$ minutes. It is obvious that had there been any anti-fibrin ferment in the incoagulable blood this should have prevented, or greatly prolonged, the clotting time of the normal sheep blood to which it had been added. The marked reduction in clotting time noted indicated that free venom was present in the incoagulable circulating blood and this quantity of venom was roughly estimated in the following manner: A sheep was bled and the normal clotting time noted. This was 20 minutes. Serial dilutions of daboia venom were prepared and added in 0.1 c.c. volume to 0.9 c.c. of this normal sheep blood, in waxed tubes, and the clotting time again noted. The result is given in Table I:—

TABLE I.

Final dilution of venom.				Coagulation time (minutes and seconds).
1-100,000	2-45
1-200,000	2-45
1-400,000	3-45
1-800,000	4-15
1-1,600,000	6-30
1-3,200,000	6-30
1-6,400,000	7-30
1-128,000,000	7-30
1-25,600,000	14
1-52,200,000	14
1-102,000,000	20
1-204,000,000	20
Control (0.9 c.c. blood with 0.1 c.c. saline)				20

It will be seen that daboia venom in a dilution of over 1 in 50 million is capable of exerting a demonstrable coagulant action on sheeps blood *in vitro*.

Next, the serum obtained by centrifugalizing the incoagulable blood of the sheep given 20 mg. of daboia venom was serially diluted and added to normal sheep blood, the clotting time of which was found to be 16 minutes. The serum, diluted one in four, reduced the clotting time from 16 minutes to 11 minutes but higher dilutions of the serum caused no reduction in clotting time. By comparing this result with the timings given in the previous table it was estimated that the venom in the blood which had reached the incoagulable stage was in this instance in a dilution of between 1 in 6 million and 1 in 12 million.

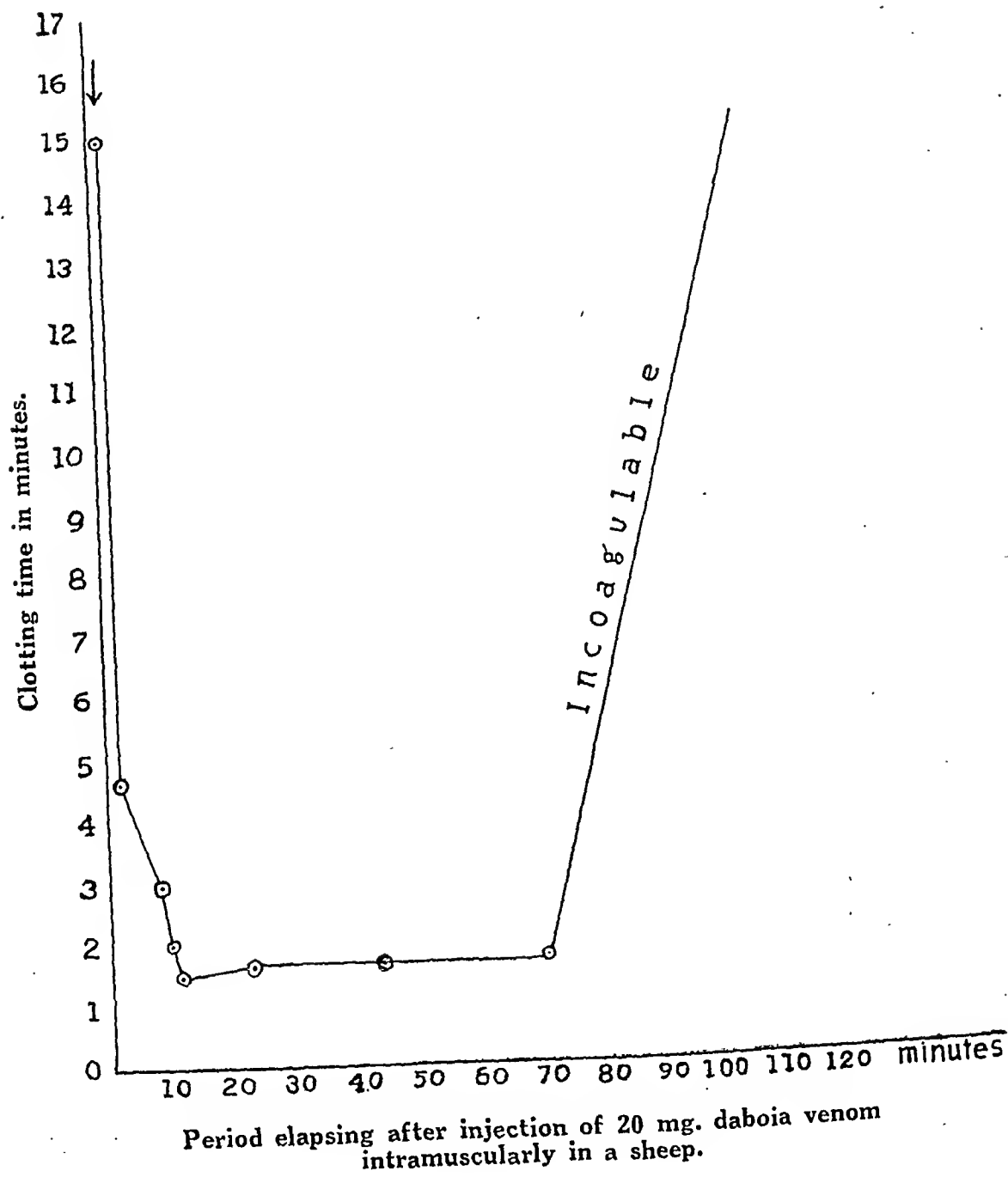
Lamb (*loc. cit.*) observed that an intramuscular injection of daboia venom was followed by decreased coagulability of blood, and that in monkeys recovery from symptoms was directly proportional to the rate of return to normal clotting time. Lamb's observations were made daily, but, so far as we are aware, recordings of clotting time at short intervals after the injection of daboia venom have not been published. We did several experiments of this nature, a typical example of the results of which is shown in the Graph (p. 176). It will be seen that a stage of increased coagulability lasting a short time precedes the stage of reduced coagulability, supporting our belief that decrease in clotting power is an end-result due to deposition of fibrin from the circulating blood. Again, Lamb (*loc. cit.*) observed that when an experimental animal was given a sub-lethal dose of daboia venom intravenously this could be followed, within a few minutes, by a lethal dose without killing the animal. Lamb's explanation of this interesting phenomenon was that the primary sub-lethal dose had liberated sufficient anti-fibrin to neutralize the coagulant effect of the subsequent dose of venom. We have confirmed Lamb's observation, but in our opinion failure to kill with the second 'lethal' dose of venom is due to the fact that the preliminary injection has already caused so much deposition of fibrin in capillaries that the second dose of venom is incapable of causing the intravascular clotting which would normally result from its administration, and it, therefore, acts in much the same way as an intramuscular injection of the venom would do. Provided then that the secondary dose has not been too great the experimental animal will escape. The alternative explanation—that anti-fibrin protects against the fibrin ferment—would suggest that the antidote to daboia venom is a further dose of the venom!

Does daboia venom contain other toxic fractions such as hæmolysin, neurotoxin and hæmorrhagin? We are of opinion that such fractions play a very minor part and are of little or no significance in causing death. In this connection it is interesting to note that, whereas cobra and other venoms have been fractionated, all attempts to fractionize daboia venom have so far failed. Admittedly, in high concentration daboia venom is capable of hæmolysing dog and horse cells in the presence of fresh complement. We have found in *in vitro* experiments that 0.01 mg. of daboia venom is feebly lytic when added to 1 c.c. of a one per cent suspension of human cells in saline, but that even 0.1 mg. of the venom will not completely hæmolyse the cells. So great a concentration of venom in the blood of a bitten person is not possible, for, according to Acton (1921), the maximum quantity of venom injected at a full bite by a Russell's viper is not more than 80 mg.

Ahuja and Brooks (1944) have described an *in vitro* method of titration of cobra antivenene which depends on the anti-hæmolytic action of the specific antivenene when added to guinea-pig cells in the presence of cobra venom. Attempts

GRAPH.

Clotting time of blood after intramuscular injection of Russell's viper (daboia) venom.



to devise a similar test for the titration of daboia antivenene have completely failed as it was found that hæmolysis was too feeble and irregular to afford any indication of the value of such anti-sera under test. Moreover, we have found that the centrifuged blood of sheep which died as the result of intramuscular injection of daboia venom is perfectly normal in appearance and without any tinge of blood indicating that hæmolysis, if any, was so little as to be of negligible importance.

That neurotoxin plays little or no active part in the toxic manifestations of daboia venom is shown by the following experiment :—

Two rabbits were anæsthetized. To the first was given an injection of 0.5 mg. of cobra venom through the cisterna magna into the cerebrospinal fluid. Three minutes after the injection, while recovering from the effects of the anæsthetic, the rabbit had a violent convulsion. It then remained paralysed and in a moribund condition till death supervened 27 minutes later. The second rabbit was given 0.5 mg. of daboia venom in a similar manner. Recovery from the anæsthetic was complete and the rabbit remained symptom-free for 37 minutes. Respiratory distress then became evident and symptoms progressed till death occurred 2½ hours later. On post-mortem examination the blood was found to be incoagulable, brain, liver and lungs were intensely congested and histological examination showed the usual clotting in capillaries with parenchymal hæmorrhages. This result is in keeping with that of Lamb (*loc. cit.*).

Experiments so far described suggest that daboia venom acts almost entirely as a rapid blood-coagulant. This is further borne out by the results of treatment in daboia poisoning at the stage when clotting in capillaries has already occurred. Thus, a delay in administering antivenene in the case of daboia poisoning is fraught with much more serious consequences than in cobra poisoning as is well exemplified by the following experiment :—

Pigeons of 300 g. to 320 g. in weight were given an intramuscular injection of 1.5 mg. of daboia venom, sufficient, in our experience, to kill in 4 to 4½ hours. At intervals of 20 minutes, treatment of these pigeons was commenced, each pigeon receiving a single dose of 2 c.c. of antivenene given intravenously—a quantity sufficient to neutralize 8 mg. of the venom *in vitro*. The result is shown in Table II :—

TABLE II.

Number of pigeons.	Daboia venom (mg.).	Antivenene after (in minutes).	Results.
2	1.5	20	One lived, one died.
2	1.5	40	Both died.
2	1.5	60	Both died.
2	1.5	80	One lived, one died.
2	1.5	100	Both died.
2	1.5	120	Both died.
2	1.5	160	Both died.
2	1.5	180	Both died.
Control (one) ...	1.5	Nil	Died.

A similar experiment conducted on exactly the same lines was carried out with cobra venom. Pigeons were given an intramuscular injection of 0.5 mg. of cobra venom, sufficient to kill, in our experience, in 4 to 4½ hours, and then treated at 20-minute intervals with a single dose of 2 c.c. of antivenene, which is able to neutralize about 4 mg. of cobra venom *in vitro*.

TABLE III.

Pigeon number.	Cobra venom (mg.).	Antivenene after (in minutes).	Results.
1	0.5	20	Lived.
2	0.5	40	Lived.
3	0.5	60	Lived.
4	0.5	80	Died (14 hours).
5	0.5	100	Lived.
6	0.5	120	Lived.
7	0.5	140	Lived.
8	0.5	160	Lived.
Control ...	0.5	Nil	Died (4½ hours).

The results of these experiments emphasize the importance of giving early and full treatment to a patient bitten by a Russell's viper, for obviously once clotting has occurred not even specific treatment can undo the harm already done. In contrast, the result with cobra venom indicates that the union between the venom and the nerve-cells is of a loose nature and easily reversible by specific treatment.

The method of assay of daboia antivenene in use at this Institute is that introduced by Anderson (1932). It consists of mixing 1 c.c. of the serum under test with 1 mg. of daboia venom, incubating the mixture for half an hour at 37°C. and injecting the mixture intravenously into a pigeon 290 g. to 320 g. in weight. If the serum is not up to titre the pigeon generally dies in 2 or 3 minutes from intravascular clotting. It is obvious that the test takes into consideration only the coagulant fraction of the venom. As, however, this fraction appears to be

all important, this *in vivo* test has stood the test of time and has proved entirely satisfactory.

Further evidence that daboia venom consists of only one important fraction is afforded by the neutralization of the action of venom by heparin, as previously reported by Ahuja *et al.* (1946). Similar results are to be expected with coumarin but difficulty in obtaining a soluble salt of this drug have so far prevented us from carrying out such tests.

When heparin is given to pigeons combined with daboia venom by the intravenous route, it is occasionally observed that some time after the injection the pigeons may show signs of daboia-venom poisoning. This observation suggested that heparin was eliminated at a quicker rate than venom. Accordingly, the following experiment was conducted:—

Pigeons were given an intravenous injection of 2 mg. of heparin followed at intervals by a single dose of 0.01 mg. of daboia venom (about 2 m.l.d.). The result is given in Table IV:—

TABLE IV.

(1) Pigeon number.	(2) Dose of heparin, mg.	(3) Dose of venom, mg.	(4) Interval between (2) and (3) in minutes.	(5) Results.
1	2	0.01	17	Lived.
2	2	0.01	30	Lived.
3	2	0.01	40	Lived.
4	2	0.01	60	Lived.
5	2	0.01	70	Died in 2 minutes.
6	Nil (control)	0.01	...	Died in 2 minutes.

Under the conditions of the experiment excess of heparin was eliminated in just over one hour. This suggests that to be of practical value in the treatment of daboia bite heparin should be given as early as possible and repeated every few hours or preferably administered by the continuous drip method.

SUMMARY AND CONCLUSIONS.

1. Experimental evidence strongly supports the view that daboia venom consists of only one fraction of any pathological importance, i.e. a strong blood-coagulant fraction.

2. Histological examination of tissues of animals dying of daboia-venom poisoning show that there is extensive deposition of fibrin in capillaries. This deprives the circulating blood of its fibrin content rendering it incoagulable. Such

incoagulable blood is itself a coagulant proving that no 'anti-fibrin ferment' exists in the venom.

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DETOXIFICATION OF KRAIT VENOM *IN VIVO* BY MEANS OF CARBOLIC SOAP SOLUTION.

BY

M. L. AHUJA,

AND

A. G. BROOKS.

(From the Central Research Institute, Kasauli.)

[Received for publication, January 9, 1948.]










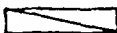




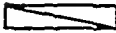
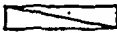







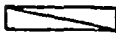



IN a previous communication Ahuja and Brooks (1945) have shown that a 5-per cent watery solution of carbolic soap (Lifebuoy) is capable of protecting pigeons against a certain lethal dose of cobra venom (*Naja naja*). This work has recently been confirmed by Christensen and Mara de Waal (1947) who found that soap solution could protect guinea-pigs against a certain lethal dose of the venoms of the African Cape cobra (*Naja flava*) and the ringhals (*Sepedon hamochates*).

This short note records our findings when soap solution is infiltrated into the area in which venom of the common krait (*Bungarus ceruleus*) has been injected. The venom was injected into the thigh-muscles of guinea-pigs and the area infiltrated one to three minutes later with one c.c. of soap solution. The protective effect of this treatment in counteracting the toxic action of krait venom is shown in the Table (see p. 182).

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TABLE.

Dose of krait venom, mg.		NUMBER OF GUINEA-PIGS. Time to death in hours.			
0.015 (No treatment)	...	 5½	 5½	 5½	 8½
0.02 (No treatment)	...	 5	 6½	 7	 8
0.02 + soap	...	 ...	 ...	 ...	 30
0.04 (No treatment)	...	 5	 4		
0.04 + soap	...	 ...	 ...	 9	 27
0.05 (No treatment)	...	 4½	 3		
0.05 + soap	...	 ...	 36		
0.06 (No treatment)	...	 3			
0.06 + soap	...	 ...	 33		
0.1 (No treatment)	...	 2½			
0.1 + soap	...	 3			



= Survived.



= Died.

NOTICE.

The following has been received for publication :—

—Editor, I.J.M.R.

21st July, 1948.

25TH ALL-INDIA MEDICAL CONFERENCE, 1948. SILVER JUBILEE SESSION—CALCUTTA.

THE Calcutta Branch of the Indian Medical Association has invited the 25th (Silver Jubilee) Session of the All-India Medical Conference to be held during the Christmas week this year (1948) in Calcutta.

Members of the profession who desire to read papers in the scientific session of the Conference are requested to send a synopsis of the paper to the undersigned by the 15th October, 1948. Original copies of all papers must reach the office by the first week of December. The synopsis must not exceed 500 words and need not contain any graph, chart or formula. Contributions shall not contain more than 3,000 words of reading matter for the Conference.

It is hoped that the profession will co-operate with the Sub-Committee in making the Scientific Session a success.

B. P. NEOGY,
Secretary, Scientific Sub-Committee.

EDITORIAL NOTE.

CLINICAL TRIALS OF NEW REMEDIES UNDER THE INDIAN RESEARCH FUND ASSOCIATION, NEW DELHI.

THE Indian Research Fund Association have recently appointed a Therapeutic Trials Committee to examine applications received by the Association for the conduct of clinical trials of new remedies and to arrange for trials of suitable remedies. This Committee will encourage and aid impartial clinical trials of biologicals, chemotherapeutic and pharmaceutical agents of Indian or Foreign origin, which offer promise in the prevention, treatment and diagnosis of disease. The Medical Research Council of the United Kingdom and the Council of Pharmacy and Chemistry of the American Medical Association have set up similar bodies in their respective countries, and it is considered that the time is now ripe for the initiation of such an organization in India also.

The Committee constituted is as follows:—

1. Dr. M. G. Kini, late Superintendent, Stanley Hospital, Madras.
2. Dr. B. Mukerji, Director, Central Drugs Laboratory, Calcutta.
3. Lieut.-Colonel Jaswant Singh, Director, Malaria Institute of India, Delhi.
4. Dr. J. C. Patel, Hony. Assistant Physician, K. E. M. Hospital, Bombay (*Secretary*).

Before undertaking trials of this nature the Committee will have to (a) decide in the first instance on the suitability of any particular drug for clinical trials and (b) collect data regarding suitability of the place and personnel who should be invited to undertake clinical trials of a particular type of drug.

The conditions under which the Indian Research Fund Association will be prepared to undertake clinical evaluation of new remedies of Indian or Foreign origin submitted by the manufacturers have been drawn up on the analogy of those adopted by the Medical Research Council of the United Kingdom and printed copies of these can be had from the Secretary, Indian Research Fund Association, New Delhi.

To carry out satisfactorily clinical trials of such agents the Association will naturally require the co-operation and help of all medical institutions in India which have facilities for such work and with this end in view these institutions are hereby requested to kindly let the Secretary, Indian Research Fund Association, know at their earliest convenience whether their institutions would be prepared to undertake clinical trials on behalf of this Association when asked to do so. Information regarding facilities available in the medical institutions in India for the conduct of clinical trials which has been invited in the questionnaire already circulated may, therefore, be furnished as soon as possible, direct to the Secretary of the Association. Detailed copies of the questionnaire can be had from the same office.

This subject is of great interest to medical men, biochemists and pharmaceutical chemists in India and their co-operation in the conduct of clinical trials will be highly appreciated.

KASAULI,

30th August, 1948.

Editor, I.J.M.R.

MODE OF CIRCULATION AND EXCRETION OF TOXINS, ANTITOXINS, VENOMS AND BACTERIA IN THE ANIMAL BODY.

BY

R. K. GOYAL, M.B., D.Sc., Ph.D., M.R.C.P., M.R.C.S., F.R.S.E.,
Assistant Director, Central Research Institute, Kasauli:

[Received for publication, January 24, 1948.]

ALTHOUGH a large amount of work has been carried out on toxins, antitoxins, venoms and bacteria, yet there being very little information available on the basic problem of the mode of circulation and excretion of these agents in the animal organism, the experiments detailed herein were carried out; diphtheria toxin and antitoxin, cobra and daboia venoms and cholera vibrio were respectively utilized in the investigation.

Diphtheria toxin.—A sample of diphtheria toxin (DTK 41) with a minimal skin-reacting dose (MRD) of 1 in 100,000 and an MLD of 0.0015 c.c. was used for injection into guinea-pigs which were bled to death at intervals. The various organs were gently washed in saline and ground up with sand for the preparation of suspensions. Serial tenfold dilutions of the suspensions were made and injected intradermally into guinea-pigs in 0.2-c.c. amounts for the detection of diphtheria toxin. When 0.5 c.c. to 1.0 c.c. of the toxin was injected intravenously, it was recovered from the blood and different organs (lung, spleen, heart, intestine, etc.) in appreciable amounts, concentration in the blood being higher than in the organs. There was a diminution in the amount of toxin in the first five hours in some guinea-pigs and not in others (the titre fell from 1 in 20,000 to 1 in 200 in the blood of one guinea-pig and remained stationary at 1 in 200 in another). No toxin was recovered from the brain. The toxin was present in appreciable amounts in the urine, but excretion through bile was either absent or negligible. Some of the results are presented in Table I.

When 0.02 c.c. of the toxin was injected intravenously into guinea-pigs, it was recovered in appreciable amounts (1 in 200 dilution) from the blood immediately after injection. This amount was sufficient to cause death of most of the guinea-pigs in about 19 hours. No toxin was recovered from the blood and organs

TABLE I.

Demonstration of toxin in different tissues and excretions in guinea-pigs injected with 0.5 c.c. to 1.0 c.c. of diphtheria toxin.

Tissues and excretions.	1.0 C.C. TOXIN INJECTED INTRAVENOUSLY.				0.5 C.C. INJECTED INTRAVENOUSLY.
	Titre 1 hour.	Titre 2 hours.	Titre 4 hours.	Titre 19 hours.	Titre 19 hours.
Blood ...	2×10^{-4}	2×10^{-4}	4×10^{-2}	2×10^{-1}	2×10^{-2}
Urine ...	10^{-3}	Undiluted (minimum)	Undiluted	...	10^{-1}
Bile*	Undiluted	10^{-1}	Undiluted	10^{-1}	...
Lung ...	10^{-1}	10^{-3} (minimum)	...	10^{-1}	Undiluted.
Spleen ...	Undiluted	Undiluted	Nil	Undiluted	Undiluted.
Supra-renal	Undiluted	Undiluted.
Liver	Undiluted	Undiluted.
Kidney	10^{-2}	...	10^{-1}	10^{-1}
Heart	10^{-2}	...	10^{-1}	Undiluted.
Small intestine ...	10^{-1}
Large intestine	10^{-1}
Brain	Nil.

* Normal bile—positive intradermal reaction with undiluted inoculum.

of a dying guinea-pig, only a small amount (undiluted inoculum only positive) was present in the urine.

In the next experiment, the effect of very small doses of diphtheria toxin on monkeys was investigated. One monkey (2,560 g.) was injected with 0.1 c.c. of the toxin intravenously; it died fairly quickly in 23 hours. Another monkey (2,400 g.) injected with 0.02 c.c. of the toxin intravenously was found dying on the 4th day; it was sacrificed and the blood and organs were tested for presence of the toxin. Small amounts of toxin were recovered from the blood and organs (kidney, lung, liver, heart), but none was demonstrable in the brain.

A rabbit injected with 0.06 c.c. (L + dose of the toxin) and dying on the third day was sacrificed. Only a small amount of toxin was found in the kidney, the other organs and blood giving negative results. Another rabbit was injected intravenously with 0.02 c.c. of the toxin. There was a concentration of 1/20 of the toxin in the blood immediately after injection. The animal was bled to death after 90 minutes; only small amounts of toxin (undiluted suspension only positive) were found in the blood and different tissues of the body.

The tissue suspensions of the animals injected a few hours previously with the toxin were washed three times with saline; no toxin was demonstrable in the washed suspensions. It can be concluded that either there was no union of the toxin with the tissues in the early stages or at best it was a loose union.

The following technique was adopted in order to determine if there is an actual union of toxin with tissues:—

Pieces of different organs from a normal animal were removed with sterile precautions and ground up in a mortar with the help of sterile sand. Normal saline solution was gradually added and the grinding was kept up till a thick suspension was obtained. A 1/100 dilution of toxin in saline was incubated with an equal amount of the suspension of organs and citrated blood for 4 to 5 hours at 37°C. Serial dilutions of the tissue suspension were prepared and tested by intradermal injections of 0.2-c.c. amounts into the epilated flanks of guinea-pigs. The skin reactions of the guinea-pigs were noted after 24 and 48 hours. The toxin was demonstrable in the different tissue suspensions in high dilutions. The incubated suspensions which had been kept in the refrigerator were then centrifuged at high speed and the supernatant fluid was discarded. The tissue deposits were re-suspended in adequate amounts of normal saline to produce very dilute suspensions, which were re-centrifuged. The suspensions were in this way washed three times. They were finally suspended in minimal amounts of normal saline solution to produce thick suspensions, which were again diluted *seriatim* and tested by intradermal injection into guinea-pigs. The presence of some toxin was still demonstrable in the washed suspensions excepting in the case of red blood cells which did not adsorb the toxin to any appreciable extent. When incubation of the toxin and thick tissue suspensions were prolonged for 24 hours in one case and 72 hours in another, the toxin was definitely destroyed by the liver but not by the kidney or citrated blood. It was of interest to note that the toxin kept in contact with kidney tissue gave a higher titre than the toxin diluted in normal saline. The kidney tissue either acted as a stabilizer or had an aggressive action on the guinea-pig's skin.

Some of the results are shown in Tables II and III:—

TABLE II.

Demonstration of adsorption and destruction of diphtheria toxin by guinea-pig tissues.

Toxin + tissues incubated at 37°C. for 24 hours.

Dilution tested.	Liver.	Blood.	Toxin control.	Dilution tested.	WASHED INCUBATED TISSUES.				
					Liver.	Spleen.	Brain.	Lung.	Kidney.
1/200	+	+	+	1/200	+	+	+	+	+
1/2,000	+	+	+	1/400	+	+	+	+	+
1/20,000	+	+	+	1/800	+	+	+	+	+
1/40,000	+	+	+	1/1,600	—	—	—	—	+
1/80,000	+	+	+	1/3,200	—	—	—	—	—
1/90,000	+	+	+						

TABLE III.

Demonstration of the destruction of diphtheria toxin by liver tissue.

Toxin + tissues incubated at 37°C. for 24 hours.

1/200 DILUTION OF TOXIN + TISSUES.			1/100 DILUTION OF TOXIN + TISSUES.			
Dilution tested.	Liver.	Kidney.	Dilution tested.	Blood.	Liver.	Toxin control.
1/200	+	+	1/20,000	+	—	+
1/600	+	+	1/30,000	+	—	+
1/1,800	+	+	1/40,000	+	—	+
1/5,400	+	+	1/50,000	+	—	+
1/16,200	—	+	1/60,000	+	—	+
1/48,600	—	+	1/70,000	+	—	+
1/145,800	—	+	1/80,000	+	—	+
			1/90,000	+	—	+

The fate of the toxin in the body of guinea-pigs immunized with diphtheria toxoid was next investigated.

The guinea-pigs had been given an injection of 5 c.c. of diphtheria toxoid at least three weeks before the injection of diphtheria toxin. One guinea-pig was injected intravenously with 1.0 c.c. of diphtheria toxin and was sacrificed shortly afterwards. No toxin could be demonstrated by intradermal injection into guinea-pigs of serial dilutions of the suspensions of organs or citrated blood.

Another immunized guinea-pig was injected intramuscularly with 1.0 c.c. of diphtheria toxin and was sacrificed after 5 minutes. The toxin was demonstrable in the muscle at the site of injection, but no toxin was recovered from the blood or viscera.

The guinea-pigs immunized with the toxoid were sacrificed 4 to 5 months after injection. The organs were ground up with sand and washed three times with saline. The immune tissues were suspended in a 1/200 dilution of diphtheria toxin and incubated at 37°C. for varying periods. The liver suspension destroyed the toxin appreciably in 5 hours, the results were slightly better after an incubation of 24 hours. The other immune organs also destroyed the toxin to some extent.

Some of the results are shown in Table IV:—

TABLE IV.

Demonstration of the action of immune tissues on diphtheria toxin.

Washed tissues + 1/200 dilution of toxin incubated for 24 hours at 37°C.

Dilution tested.	Liver.	Dilution tested.	Kidney.	Heart.	Spleen.
1/20	+	1/800	+	+	+
1/40	+	1/1,600	+	+	+
1/80	+	1/3,200	+	+	+
1/160	+	1/6,400	+	+	+
1/320	±	1/12,800	±	+	+
1/640	±	1/25,600	±	+	+
1/1,280	±	1/51,200	—	+	±
1/2,560	—				

Diphtheria antitoxin.—On intravenous injection of 1,500 units of diphtheria antitoxin into guinea-pigs, 85 to 90 units of antitoxin were recovered immediately afterwards from the serum and 0.4 to 0.8 units were recovered from the organs and urine in two tests carried out immediately after the injection and after a period of 90 minutes. No antitoxin was recovered from the suspension of organs washed three times with saline. After three days, only 10 units of the antitoxin were recovered from citrated whole blood but no antitoxin was demonstrable in tissue suspensions or urine.

For titration of the antitoxin content of serum and suspension of organs, the Lr/100 doses of the toxin were mixed with varying dilutions of the antitoxin-containing material. As the mixtures were to be injected intradermally in exactly 0.2-c.c. amounts, mixtures for 2 to 10 animals were prepared depending on the amount of material available. Normal saline solution was added to make up the total volumes to certain pre-determined higher multiples of 0.2 c.c., i.e. the mixtures for two were made up to 0.4 c.c. and mixtures for ten up to 2.0 c.c. A large number of preliminary ranging experiments had to be carried out at widely-spaced intervals to arrive at the approximate titre of the antitoxin, after which more closely-spaced dilutions were prepared and tested *in vivo*.

Some of the results are presented in Tables V and VI :—

TABLE V.

Antitoxin content of the tissues and excretions of guinea-pigs injected with 1,500 units of antitoxin.

Guinea-pigs sacrificed up to 90 minutes after injection.

Unitage tested for.	Dilution of suspension.	Dose of toxin, c.c.	Volume.	Heart.	Lung.	Kidney.	Liver.	Spleen.	Urine.	Bile.
0.1	Undiluted	0.00058	0.2	—	—	—	—	—	—	..
0.2	1/2	"	"	—	—	—	—	—	—	+
0.4	1/4	"	"	—	—	—	—	—	—	+
0.8	1/8	"	"	++	+	++	—	++	—	+
1.6	1/16	"	"	++	...	+	+

TABLE VI.

Antitoxin content of the serum of guinea-pigs injected with 1,500 units of antitoxin.

Guinea-pigs sacrificed immediately after injection.

Unitage tested for.	Toxin, c.c.	Amount of serum.	Volume.	Result.
50	0.00058	$\frac{1}{50 \times 100}$	0.2	—
60	"	$\frac{1}{60 \times 100}$	"	—
70	"	$\frac{1}{70 \times 100}$	"	—
80	"	$\frac{1}{80 \times 100}$	"	—
90	"	$\frac{1}{90 \times 100}$	"	+
100	"	$\frac{1}{100 \times 100}$	"	+

Daboia venom.—Twenty mg. of daboia venom were injected intravenously into a guinea-pig. It was bled from the heart immediately after the injection; the animal died within 2 minutes. The presence of venom was determined by injecting 0.5 c.c. of double dilutions of the citrated blood intraperitoneally into mice and intradermal injections of the same dilutions of the blood were given into the epilated flanks of guinea-pigs. The suspensions from different organs were tested similarly for the presence of venom. It was demonstrable in a 1/16 dilution of the citrated blood by the guinea-pig intradermal test and in a 1/8 dilution by the mouse lethal test. No venom was demonstrable in the different organs, secretions or excretions, viz. lung, liver, spleen, bile and urine. The experiment was repeated twice with similar results. The minimal skin-reacting dose of daboia venom in a guinea-pig being 0.05 mg. and the MLD for a mouse injected intraperitoneally, 0.02 mg., it is evident that a considerable amount of venom was destroyed in the body immediately after injection.

The most sensitive tests for the detection of daboia venom are the *in vitro* tests, i.e. coagulation and hæmolytic tests. The coagulative property of the venom could not be made use of as fairly high dilutions of normal tissue suspensions caused clotting of calcified citrated sheep blood. The hæmolytic test was performed by,

mixing double dilutions of daboia venom with 3-per cent suspensions of goat-cells + 0.2 c.c. of egg-yolk solution (the yolk of a fresh egg emulsified in 250 c.c. of normal saline solution and kept in a refrigerator). The mixtures were incubated at 37°C. for one hour and then left overnight at room temperature. The presence of the venom could be detected in a dilution of at least 1/500 of a mg. The hæmolytic test revealed the presence of only a negligible amount (up to 1/10 dilution) of venom in different tissues.

Similar results were observed when 0.5 mg. of venom was administered intracardially.

The intravenous injection of fairly big doses of daboia venom resulted in very early death of the guinea-pigs. In order to allow the venom to act more slowly, intramuscular injections were given. Three guinea-pigs were injected intramuscularly with 2 mg. to 4 mg. of venom and were killed at intervals varying from 25 minutes to 3½ hours respectively. Small amounts of venom (1/8 to 1/64 dilution) were demonstrable at the site of injection. Either no venom or a minute trace of it (undiluted suspension positive) was found in the blood and other tissues.

The rapid disappearance of the venom from the body might be due to the destructive action of normal tissues on the venom. When tissue suspensions were incubated with 2 mg. of venom solution up to 4 hours at 37°C. there was only a slight destruction of the venom. When the incubation of thick tissue-suspensions + 3 mg. of venom solution was prolonged for 22 to 23 hours at 37°C., there was a marked destruction of venom (hæmolysis up to 1/8 dilution only).

Cobra venom.—An intracardial injection of 2.0 mg. of cobra venom into a guinea-pig caused death in two minutes. A fair amount of venom was demonstrable in blood by the hæmolytic test (1/500 dilution), but none was found in tissue suspensions. The minimal hæmolytic dose of this venom was 1/500th of a milligram.

Another guinea-pig was injected subcutaneously with 5.0 mg. of cobra venom. It was sacrificed after 30 minutes. A fair amount of venom (1/500 dilution) was demonstrable by the hæmolytic test at the site of injection, but none was found in the blood or tissue suspensions.

Cholera vibrio.—On intradermal inoculation of 2 mg. of agglutinable vibrios into guinea-pigs, the number of vibrios in blood circulation rose from 200 in 30 seconds to at least 200,000 in 15 minutes. The bacteraemia persisted irregularly in different guinea-pigs, began to decrease from the 11th hour onwards and disappeared in 16½ hours. A secondary rise was noticeable after 24 hours in some of the guinea-pigs injected.

The invasive power of the vibrios was studied in 21 guinea-pigs. It has not been considered advisable to give details of the bacterial count in all the injected animals but certain illustrative cases only have been mentioned.

Different kinds of vibrios (two strains of *V. cholerae* sub-type Inaba, one recently isolated from a case and the other an old laboratory strain and an NAG vibrio) were injected subcutaneously into guinea-pigs in 10-mg. doses. All vibrios excepting the Inaba strain were recovered within 24 hours from almost all the organs examined (skin, lymph glands, spleen, liver, kidney, lung, etc.). The Inaba strain was

recovered from fewer organs (skin, liver and urine in one case). No vibrios could be recovered from any organ 72 hours after inoculation.

After an intradermal injection of 2 mg. of *V. cholerae* most of the organs gave positive cultures 24 hours after infection. Skin and spleen only were positive after 48 hours but no vibrios could be recovered from any organ after 3 days.

It might be pointed out that Laporte and Goyal (1936) found that the typhoid bacillus injected in non-lethal doses persisted in the animal organism for several weeks.

The case NAG vibrio and water vibrios gave results almost similar to those obtained with agglutinable vibrios. The water vibrio was recovered from comparatively more organs (skin, lymph glands and spleen) than the case strain Inaba type of *V. cholerae*.

These experiments were carried out in a number of animals, 4 to 9 guinea-pigs being used in the case of each type of vibrio; it has not been considered necessary to give details of the findings for each individual guinea-pig.

In one rabbit no vibrios could be recovered from 0.5 c.c. of blood after intradermal injection of 2 mg. In a second rabbit, the number of vibrios rose to 2,000 per c.c. within an hour, decreased to 200 per c.c. of blood in the second hour and no vibrios were recoverable from the 3rd hour onwards. Positive cultures were obtained from the skin and lymphatic glands 48 hours after injection.

The rate of destruction of vibrios in immunized guinea-pigs was next studied. Seven days after an intradermal injection of 2 mg. of agglutinable vibrios, the guinea-pigs were re-injected in the same way. The blood cleared within 3 to 4 hours. The animals were sacrificed after 4 hours. Vibrios were recovered from the infected skin only in one case and from the spleen alone in another guinea-pig.

DISCUSSION.

In the absence of detailed information about the mode of circulation of toxins and organisms in the animal body, it is not scientifically possible or advisable to carry out investigations or to devise suitable methods for combating the effects of these noxious agents. For this type of work one should be in possession of suitable methods for the detection of minute quantities of toxins and the isolation of organisms from contaminated sources. The intracutaneous titration of diphtheria antitoxin was introduced as late as 1921 by Glenny and Allen (1921). Prior to this only the lethal test was available for the detection of comparatively large amounts of diphtheria antitoxin. Glenny and Hopkins (1923) gave details of the excretion of diphtheria antitoxin in passively immunized guinea-pigs, but there is very little information about the presence and mode of union of antitoxin with tissues. Similarly, only certain isolated experiments have been published about the action of tissues on diphtheria toxin. Wadsworth and Hoppe (1931) reported that diphtheria toxin was not destroyed by the adult guinea-pig's cardiac muscle.

After the injection of diphtheria toxin into non-immune animals, appreciable amounts of the toxin could be recovered from the blood and different organs for varying periods, normal liver tissue was shown to play an important rôle in the destruction of the toxin. When big doses of diphtheria toxin were injected into immunized guinea-pigs, no toxin could be demonstrated in the blood or viscera. The disappearance of the toxin was evidently partially due to its union with the circulating antitoxin, but its destruction was to a certain extent also due to the existence of tissue immunity. Whether the tissue immunity was due to the presence of the firmly united antitoxin or there was an inherent change in the tissues could not be determined.

The workers concerned with raising antivenenes advocate the injection of venoms by the subcutaneous route along with tapioca, tannin, etc., under the belief that a depôt of venom is created at the site of injection, from which the venom is gradually absorbed. The present work shows that most of the injected venom is quickly destroyed. Experiments carried out by the writer but not reported here show that there is no need to waste large amounts of venom by administering it subcutaneously to horses for raising antisera; the same results can be achieved by giving much smaller doses by the intravenous route.

SUMMARY.

The following observations were made during an investigation into the mode of circulation and excretion of toxins, antitoxins, venoms and cholera vibrio:—

1. The intravenous injection of diphtheria toxin into guinea-pigs produced transient toxæmia. The toxin was found in the urine in appreciable amounts but excretion through bile was either absent or negligible.

2. After the administration of minute doses of diphtheria toxin leading to death after a fairly long period, no toxin could very often be demonstrated in most of the organs of these animals.

3. The toxin either did not combine with the tissues or there was at best a loose union in the early stage of toxæmia.

4. The liver tissue was found to play an important part in the destruction of diphtheria toxin.

5. No toxin could be demonstrated in the tissues of immunized guinea-pigs dying from the injection of big doses of toxin.

6. After administration of big doses of diphtheria antitoxin, very small concentrations of it were found in the tissues, and only in the early stages. No union of antitoxin with the tissues was discernible.

7. After administration of big doses of daboia and cobra venoms to guinea-pigs either no venom or negligible amounts were found in the tissues.

8. Cholera vibrio injected in non-lethal doses into guinea-pigs disappeared completely from the system in three days. This was in marked contrast to the behaviour of the typhoid bacillus.

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ON THE NATURE OF PENICILLIN BACTERIOSTASIS.

Part I.

NUCLEIC ACID ANTAGONISM OF PENICILLIN BACTERIOSTASIS.

BY

(Miss) MARIAM GEORGE,

AND

K. M. PANDALAI.

*(From the Department of Biochemistry, Indian Institute of Science,
Bangalore.)*

[Received for publication, February 2, 1948.]

SEVERAL attempts have been made to study antagonists to drug action, but some productive results seem to have been achieved only in the case of the sulphonamides and to a less extent in the case of the acridine derivatives (McIlwain, 1941). Among the many theories to explain the mechanism of drug action developed in recent years the concept (Fildes, 1940) that drugs act by metabolic interference with the action of the pathogens has gained much prominence. Another interesting explanation is that the drugs 'poise' the oxidation reduction potential in the immediate environment of the cells at a level which precludes the normal functioning of one or more desmolytic enzyme systems (Henry, 1943). It has been shown (Dubos, 1945) that the bacteriostatic dyes exert their action by ionic exchange reactions, the dyes replacing similarly charged ions already present in the cells.

These suggest the possibility that penicillin might also exert its action by interfering with a metabolite essential for the parasite's growth, exercising this interference effect by oxidizing substance which requires to be reduced or by molecular combination forming an inactive product or by competition for an enzyme associated with the utilization of the metabolite by the parasite. It becomes necessary, therefore, to study the connection between the physiological features of the drug action and some aspect of the metabolic process in the parasite cells. Accordingly, we studied the influence of substances, such as nucleic acid, thiamine,

riboflavin, nicotinic acid, tryptophane, uracil, etc., on the growth of typical parasites in presence of penicillin. These substances besides being essential growth-factors for several parasites are also known to be parts of the prosthetic groups of enzymes, playing important rôles in cellular respiration. McIlwain (*loc. cit.*) has demonstrated nucleic acid antagonism of the antibacterial action of acridine derivatives and Krampitz and Werkman (1947) have shown that penicillin interferes with the metabolism of the nucleic acids or nucleotides in the case of *Staphylococcus aureus*. In this paper the results obtained with nucleic acid which is a major constituent of the bacterial cell are given.

EXPERIMENTAL.

Twenty-four-hour old cultures of the organisms were used in one loopful in sterile culture tubes in duplicates containing a total volume of 10 c.c. including nutrient broth and the test substance added in requisite amounts. The tubes were observed after 24 hours' incubation at 37°C. and the turbidity in the tubes due to bacterial growth observed. The results given in Table I show that nucleic acid when present in broth does not act either as a growth-promoting or growth-inhibiting substance, but has a very pronounced antagonizing action on penicillin bacteriostasis of *Staph. aureus*:—

TABLE I.

Showing the influence of penicillin and nucleic acid on the growth of *Staph. aureus*.

Penicillin units/c.c.	Growth in broth.	Nucleic acid.	Growth in broth.	Penicillin units/c.c. and nucleic acid.	Growth in broth.	Control in broth.
0.3	—	1/2,000	+++	0.3	+	+++
				0.2	++	
				0.1	++	
0.2	—	1/5,000	+++	0.3	+	
				0.2	++	
				0.1	++	
0.1	+	1/10,000	+++	0.3	+	
				0.2	++	
				0.1	++	
0.05	+	1/20,000	+++	0.3	+	
				0.2	+	
				0.1	+	

+++ indicates very good growth; ++ indicates fairly good growth; + indicates scanty growth; and — indicates no growth.

In the presence of added nucleic acid the penicillin either did not side-track or inactivate the cell nucleic acids, or even if it did the organisms could still thrive on the external source of nucleic acid thus nullifying the bacteriostatic effect of the drug. Whether penicillin forms inactive complexes with the cell nucleic acids or only side-track reversibly vital metabolic essentials like nucleic acids from their normal function has to be made clear. Such complexes, even if formed, need not necessarily be precipitated but can act by reducing the effective concentration of penicillin in solution, thus effecting a net withdrawal of the penicillin from the bacteria.

That penicillin bacteriostasis of *Staph. aureus* is reversible by nucleic acid was made clear from the following experiments: Cultures which were treated with penicillin in sub-lethal concentrations received at different intervals additions of nucleic acid and were incubated as usual. These tests revealed as is seen in Table II that organisms exposed to penicillin and became non-viable could actually be rendered viable by exposure to nucleic acid. Even after 24 hours' contact with the drug in a tube into which one loopful of the organisms was inoculated in 10 c.c. of broth could get revived and restored to normal growth by receiving additions of nucleic acid.

TABLE II.

Showing the reversibility of penicillin bacteriostasis of *Staph. aureus* by added nucleic acid.

Penicillin units/c.c.	Nucleic acid added after		Penicillin control.	Nucleic acid control.	Broth control.
	3 hours.	6 hours.			
0.1	1/1,000 ++	++	+	+++	+++
	1/2,500 ++	++		+++	
	1/5,000 ++	++		+++	
0.2	1/1,000 ++	++	-	+++	
	1/2,500 ++	++		+++	
	1/5,000 ++	++		+++	
0.3	1/1,000 ++	++	-	+++	
	1/2,500 ++	++		+++	
	1/5,000 ++	++		+++	

Reversibility of penicillin action appeared to be a common feature in the case of other Gram-positive organisms, for, as is seen in Tables III and IV, nucleic acid reversed the inhibitions of *Streptococcus viridans* and *B. subtilis* by penicillin when added immediately after inoculation (Table III) and after certain intervals of times (Table IV):—

TABLE III.

Showing that nucleic acid antagonism of penicillin action is a common feature of Gram-positive organisms.

Organisms.	Growth in the presence of penicillin units/c.c.			Growth in the presence of nucleic acid.			Growth in the presence of penicillin and nucleic acid.			Control.
	0.3	0.2	0.1	1/1,000	1/2,000	1/5,000	0.3 } 0.2 } 0.1 } 1 1,000	0.3 } 0.2 } 0.1 } 1 2,000	0.3 } 0.2 } 0.1 } 1 5,000	
<i>Staph. aureus</i> ...	-	-	+	+	+	+	+++	+++	+++	+
<i>Strept. viridans</i> ...	-	-	-	+	+	+	+++	+++	+++	+
<i>B. subtilis</i> ...	-	-	+	+	+	+	+++	+++	+++	+

TABLE IV.

Showing that reversibility of penicillin action by nucleic acid is a common feature of Gram-positive organisms.

Organisms.	Growth in the presence of penicillin units/c.c.				Growth in penicillin treated tubes receiving additions of nucleic acid at different intervals.						Control.
	0.3	0.2	0.1	0.01	After 3 hours.			After 6 hours.			
					1/1,000	1/2,000	1/5,000	1/1,000	1/2,000	1/5,000	
<i>Staph. aureus</i>	—	—	—	+	+	+	+	+	+	+	+
<i>Strept. viridans</i>	—	—	—	—	+	+	+	+	+	+	+
<i>B. subtilis</i>	—	—	—	+	+	+	+	+	+	+	+

TABLE V.
Showing the absence of any effect of nucleic acid on the penicillin inhibition of Gram-negative organisms.

Organisms.	Growth in the presence of penicillin units/c.c.			Growth in the presence of nucleic acid.			Growth in the presence of penicillin and nucleic acid.			Control.
	12	15	20	1/1,000	1/2,000	1/5,000	12 15 20 1/1,000	12 15 20 1/2,000	12 15 20 1/5,000	
<i>E. coli</i> ...	+	-	-	+	+	+	+++	+++	+++	+
<i>E. typhosa</i> ...	+	-	-	+	+	+	+++	+++	+++	+
<i>B. dysenteriae</i> (<i>Shigella</i>).	+	-	-	+	+	+	+++	+++	+++	+

These results show that the Gram-positive cocci and bacilli behave uniformly both in their capacity to grow in the presence of nucleic acid and penicillin and in the capacity to get revived after having become non-viable by contact with penicillin.

In another series of experiments, it was sought to find out whether the Gram-negative organisms which are usually inhibited only by very high concentrations of penicillin will also behave similar to the Gram-positive organisms when allowed to grow in the presence of penicillin and nucleic acid. The results obtained using *E. coli*, *E. typhosa*, and *B. dysenteriae* (*Shigella*) are shown in Table V.

The complete absence of growth-influencing or inhibition-reversing properties in the case of nucleic acid on Gram-negative organisms made it desirable to study the effect of penicillin and nucleic acid on a Gram-positive organism which under the ordinary conditions is penicillin insensitive. *Streptococcus faecalis* was chosen for this purpose and the results are given in Table VI:—

TABLE VI.

Showing the influence of penicillin and nucleic acid on the growth of *Strept. faecalis*.

Growth in the presence of penicillin, units per c.c.		Growth in the presence of nucleic acid.		Growth in the presence of penicillin and nucleic acid.		Control.
5	—	1/1,000	+	$\left\{ \begin{array}{c} 3 \\ 2.5 \\ 2 \\ 1 \end{array} \right\}$	$\left\{ \begin{array}{c} 1/1,000 \\ - \\ - \\ \pm \\ + \end{array} \right\}$	+
3	—	1/2,000	+	$\left\{ \begin{array}{c} 3 \\ 2.5 \\ 2 \\ 1 \end{array} \right\}$	$\left\{ \begin{array}{c} 1/2,000 \\ - \\ - \\ \pm \\ + \end{array} \right\}$	+
2.5	—	1/5,000	+	$\left\{ \begin{array}{c} 3 \\ 2.5 \\ 2 \\ 1 \end{array} \right\}$	$\left\{ \begin{array}{c} 1/5,000 \\ - \\ - \\ \pm \\ + \end{array} \right\}$	+
2	±
1	+

These results show that the Gram-positive organisms require a minimum inhibiting concentration of penicillin much nearer to that required by the penicillin-sensitive Gram-positive organisms than to the penicillin-insensitive Gram-negative organisms. These findings undoubtedly show that penicillin bacteriostasis is associated with some important action on the cellular nucleic acids of the pathogens and their Gram-staining nature. These aspects are treated in Part II of this paper (George and Pandalai, 1948—see p. 205, this issue).

DISCUSSION.

Penicillin seems to exert on the susceptible cells a limited specific injury completely reversible in the case of Gram-positive organisms by nucleic acid probably achieving this by blocking the enzymes which subserve to the utilization of nucleic acid in some specific form, essential for the metabolic process of the parasites. Not only nucleic acid by its mere presence allow organisms normally inhibited by penicillin to grow, but it also allows an already inhibited culture to grow and multiply in the normal way. The interesting point of the specificity in the growth-influencing and inhibition-reversing properties possessed by nucleic acid on penicillin-sensitive Gram-positive organisms make it necessary to assume that metabolic reaction pathways are different more or less in the case of Gram-positive and Gram-negative organisms. The reversibility of bacteriostasis shows that important growth essentials or biochemical agents involved in cellular processes are only reversibility side-tracked as a consequence of which instantaneous death of the cells never results but only temporary inhibition of cell division and multiplications. The view of several workers that penicillin appears to interfere with a metabolic function involved in the early stages of bacterial growth has thus been confirmed. It is also clear that of the important cellular agents involved in penicillin bacteriostasis, nucleic acids are of paramount importance.

SUMMARY.

1. Nucleic acid possesses a pronounced antagonistic effect on penicillin bacteriostasis and the action of penicillin is rendered reversible when nucleic acid is added to inhibited cultures.

2. While the growth-influencing and inhibition-reversing properties of nucleic acid is a general feature as far as the Gram-positive *cocci* and *bacilli* are concerned it has no such influence at all on Gram-negative organisms even in high concentrations.

3. It is concluded that metabolic interference is the prime factor in the bacteriostasis brought about by penicillin and one of the cellular agents which penicillin inactivates and which is an inevitable essential factor in the normal metabolism of the pathogens seems to be the nucleic acids.

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ON THE NATURE OF PENICILLIN BACTERIOSTASIS.

Part II.

RELATION BETWEEN PENICILLIN ACTION AND THE GRAM-STAINING CHARACTERISTICS OF CERTAIN PATHOGENS.

BY

(Miss) MARIAM GEORGE,

AND

K. M. PANDALAI.

(From the Department of Biochemistry, Indian Institute of Science, Bangalore.)

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THE antibacterial action of penicillin is in general selective to Gram-positive organisms, cocci and bacilli, and to certain Gram-negative organisms like the gonococci and meningococci spirochaetes, etc. This peculiar selectivity in the action of penicillin has not been adequately explained. It has been shown that a few Gram-negative organisms, such as *E. coli*, *E. typhosa*, etc., which under ordinary conditions are insensitive to penicillin are really inhibited when the latter is used in high concentrations (George and Pandalai, 1946). This aspect led us to study the changes in certain pathogens when they were left in contact with subminimal inhibiting concentrations of penicillin. Gardner (1940) has noted striking changes in the shape of the bacteria after exposure to varying concentrations of penicillin. Elongation and swelling of the bacterial cells in *C. relchii*, *E. typhosa*, *V. cholera* and even *E. coli*, spherical enlargement of cells of *Staph. aureus*, enlargement of cells and increase in the length of chains in the case of streptococci were among the changes he noted when dilutions of penicillin well above those which were completely inhibitory to their growth were used. Besides these purely morphological changes no emphasis seems to have been made on the Gram-staining reactions of the organisms when they are left in contact with penicillin, and hence it was

thought worth while to record certain observations we had made on the Gram-staining changes which preceded bacteriostasis by penicillin in the case of certain pathogens.

EXPERIMENTAL.

The organisms were subcultured in nutrient broth and incubated at 37°C. for 18 hours. Subcultures were made from these into several sterile tubes containing the required quantities of the broth and penicillin diluted in broth to give the required concentrations. One loop of the 18-hour old culture was used as the inoculum in each tube. All the tubes were incubated at 37°C. and the tubes were taken after definite intervals, centrifuged, washed and the organisms in the sediment stained according to the Gram-staining technique and examined. The results are recorded in Table I:—

TABLE I.

Showing the staining characteristics of E. coli, E. typhosa, B. dysenteriae (Shigella) in contact with penicillin during different intervals of time.

Organisms.	Growth in the presence of penicillin units per c.c.			Staining after									Control after		
				6 hours.			12 hours.			24 hours.			6 hours.	12 hours.	24 hours.
	S	10	12	S	10	12	S	10	12	S	10	12			
<i>E. coli</i> ...	++	++	±	P	P	P	P	P	P	P	P	P	N	N	N
<i>E. typhosa</i> ...	++	++	+	P	P	N	P	P	P	P	P	P	N	N	N
<i>B. dysenteriae</i> (<i>Shigella</i>).	++	++	—	P	P	P	P	P	O	P	P	O	N	N	N

P = Gram-positive. N = Gram-negative. O = Not stained.

It is seen that all the three organisms get definitely Gram-reversed and attain the power of retaining the violet stain. These Gram-reversed organisms, when centrifuged, washed well and subcultured as usual into nutrient broth, incubated and examined again after 24 hours by the Gram-staining technique, revealed, however, that the organisms had lost the acquired property of retaining the violet stain having uniformly reverted themselves to the original Gram-negative type.

In another series of experiments the treated cultures were centrifuged, washed and the sediment suspended in the original volume of the broth and one loop from these each was used side by side with one loop from the untreated control culture

to ascertain the minimum inhibiting concentrations of penicillin for the treated organisms. Results are presented in Table II:—

TABLE II.

Showing that the minimum inhibiting concentrations of penicillin required for the Gram-reversed organisms are higher than those required for the untreated strains.

Penicillin units/c.c.	<i>E. coli.</i>		<i>E. typhosa.</i>		<i>B. dysenteriae.</i>	
	Treated.	Untreated.	Treated.	Untreated.	Treated.	Untreated.
5	+	+	+	+	+	+
7	+	+	+	+	+	+
9	+	+	+	+	+	+
10	+	+	+	+	+	±
15	+	—	+	—	+	—
20	+	—	+	—	+	—

+ indicates viable growth. — indicates absence of growth.

These results reveal the fact that the treated organisms are not inhibited by the amount of penicillin which inhibits the untreated organisms. Indeed they need higher concentrations. This is probably due to the acquiring of resistance to the drug due to contact with it. The question of penicillinase production with resultant inactivation of penicillin cannot explain the difference in all the cases even if it may be partly responsible in the case of *E. coli*. The subcultures from the penicillin-treated tubes behaved exactly as the untreated parent strains in morphological features.

Such Gram-reversals when in contact with penicillin happened also in the case of Gram-positive organisms like *Staph. aureus*, *Strept. haemolyticus* and *Pneumococcus* type I. It was noticed that these organisms after becoming Gram-reversed undergo further change to the original Gram-positive stage either by efflux of time or by contact with added nucleic acid. The organisms were grown in appropriate media. (For *Strept. haemolyticus* and *Pneumococcus* nutrient broth was enriched with rabbit's blood in the presence of penicillin in subminimal inhibiting concentrations.) In 18 hours even the tubes containing 0.01 units of penicillin per c.c. showed definitely Gram-negative organisms. The contents of each tube were carefully divided into two portions in sterile tubes and one of each received addition of nucleic acid in 1/1,000 concentration, while the other was allowed to remain as such. All the tubes were examined after 18 hours' incubation. It was observed, as would be seen from Table III which gives the results of the

STUDIES ON RURAL WATER-SUPPLIES.*

BY

K. SUBRAHMANYAN,

AND

T. R. BHASKARAN,

(Section of Sanitary Engineering)

WITH

STATISTICAL TREATMENT

BY

C. CHANDRA SEKAR.

(Section of Statistics).

(From the All-India Institute of Hygiene & Public Health, Calcutta.)

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Part I.

INTRODUCTION.

THE vast majority of people in India live in rural areas and do not enjoy the benefits of protected public water-supplies. They use water from rivers, tanks, canals, wells and tube-wells. Their state of health will depend to a certain extent on the wholesomeness and quality of the water they use. Widespread water-

* The studies and observations on which this paper is based were conducted with the generous support of the International Health Division of the Rockefeller Foundation and with the facilities afforded by the Health Unit, Singur, in the field.

borne epidemics may not occur in the rural areas owing to the multiplicity of small sources used by the people. The consequences of contamination of a particular source of water in the rural areas may, therefore, not be so spectacular as in an urban community served by a piped supply from a single large source.

The standards of purity laid down by the British Ministry of Health (1940) and the U.S. Treasury Department for public water-supplies are based on numerical estimate of *coliform* organisms, although the basis on which numerical limits were framed is not quite clear. More recently, Kehr and Butterfield (1943) have traced a relation between typhoid organisms and the *coliforms* in actual typhoid epidemics in certain cities and have justified the use of the most probable number of *coliform* organisms as the index of purity of a water-supply.

Most surface waters in rural areas are exposed to gross contamination and it is, therefore, difficult to accept them as satisfactory for a community. In such cases a high *coliform* count is to be expected and the actual numbers are of little importance as the probability of contamination is so obvious. However, in the case of ground-water from wells of various kinds, and especially tube-wells, the chances of contamination are somewhat less. Contamination of water in these sources may occur either directly from the surface or below the surface through the soil. The extent of contamination of the first kind will depend on the chances of exposure of water and that of the second kind will depend on soil conditions and proximity of sources of pollution. The latter has been emphasized, and perhaps over-emphasized by sanitarians in all countries. Caldwell and Parr (1937) and Dyer *et al.* (1945) have found that in sandy or clayey soils free from fissures, bacteria from such an obvious source of pollution as the borehole latrine do not travel more than 10 ft. to 25 ft. in the direction of flow when the rate of withdrawal of ground-water is not high, and therefore the chances of contamination of wells subject to such usage as may be expected in rural areas are not so great as has been believed in the past. The studies described in this paper are a sequel to the investigations carried out by Dyer *et al.* (*loc. cit.*). These were conducted with the aid of a generous grant from the Rockefeller Foundation from 1943 to 1946. The results are based on field observations and laboratory findings on a variety of actual sources of water-supply used by a rural community of about 27,000 people forming part of the Health Unit at Singur in West Bengal.

There are many aspects of the problem of rural water-supplies and only some have been covered in these studies. The quality of water available in various locations and depths and the effect of different factors—seasons, sanitary conservation of the source, frequency of usage and efficiency of maintenance service,—on the bacteriological quality of water obtained from tube-wells have been studied. The necessity for an organized maintenance service for tube-wells was realized early and data have been collected on the nature and cost of maintenance.

An attempt has also been made to work out practicable bacteriological standards for rural tube-wells. Whether there is any proven epidemiological justification for laying down these standards in terms of *coliform* numbers is a question that cannot be answered from these studies. The results only throw light on what standards are practicable with reasonable care and maintenance in rural areas for a water that is proved to be uncontaminated by surface water. We also know that

information to that effect and information was collected under the following heads in regard to every repair :—

1. Union Board—village—number of well.
2. Agency, date and time of report.
3. Date and time of repair.
4. Time between the well going out of order and receipt of information.
5. Number of days the well was out of order before it was repaired.
6. Number of days between the receipt of information and effecting repair.
7. Time taken for repair.
8. Kind of pump used.
9. Nature of repair.
10. Cost of materials used in repair.
11. Whether the fitter could repair alone or needed the help of another hand.

The Singur Health Centre maintains data on the cost of maintenance of all public tube-wells in the 4 union boards and also the number of repairs effected, etc.

The tube-wells were classified into three classes from consideration of sanitary conservation, and those have been compared for bacteriological quality. Private tube-wells which have a satisfactory apron and a waste-water drain at least 5 ft. long in good condition are classified as A. Public tube-wells of this class are called A_p. Tube-wells having only a satisfactory apron but no drain are classed as B in the case of private tube-wells and B_p in the case of public tube-wells. Those which have neither an apron nor a drain for waste-water are classified as C (for private tube-wells) and C_p (for public tube-wells). A further distinction is made between wells which have a satisfactory cement plug at the base of the pump and those which have none.

A similar sanitary classification of shallow open wells has also been made. As a rule none of these wells is fitted with a cover or a pump. Water is drawn from some of these wells by a pulley and bucket, while in others it is drawn by buckets let down by hand.

Open wells have also been classified into the following five sanitary categories:—

- D—parapet, apron and drain satisfactory.
- E—parapet and apron satisfactory but drain for waste-water less than 5 ft. long or broken or not provided.
- F—parapet and drain satisfactory but apron unsatisfactory.
- G—only parapet or apron or drain satisfactory.
- H—parapet, apron and drain all unsatisfactory.

It may be remarked that open wells are mostly privately owned. They are less popular than tube-wells for drinking water-supply.

Sampling design.—The programme of sampling of a certain number of sources was statistically planned so as to obtain a picture of the changing water quality in the different types of wells in the experimental area. Since all tube-wells could not be sampled a selection was made from among them ensuring representation of the whole area. For this purpose Singur has been divided into 4 areas and Balarambati into 2 and as far as possible an equal number of wells from the different categories was chosen at random from these areas. This restricted the selection

of wells under each category to the smallest number available in the different categories. It was considered that every well should be examined bacteriologically at least once a fortnight in order to get a continuous picture of the quality of water and to correlate water quality with the state of health of the users.

TABLE I.

Number of wells chosen for sampling.

	TUBE-WELLS.							Open wells.	Tanks.
	Private.			Public.			Total.		
	A	B	C	A _p	B _p	C _p			
Singur ...	20	12	Nil	12	28	12	84	20	1
Balarambati ...	10	10	3	8	16	3	50	24	3

All the 182 sources were sampled once a fortnight during the first one year. Later on the sampling schedule was modified in the light of the first year's findings so as to include fewer tube-wells for observation and more frequent observation of the open wells.

Methods of bacteriological and chemical examination of water samples.—The bacteriological samples were examined for total agar count at 37°C., presumptive *coliform* count on MacConkey broth (37°C.), pH, temperature and electrical conductivity. When the *coliform* count exceeded 10 per 100 e.e., the organisms were also differentiated by the 'IMVIC' reactions. The dilutions chosen for the presumptive count were as follows:—

Tube-wells.—50 e.e.—1 flask; 10 e.e., 1 c.c. and 0.1 c.c.—3 tubes each.

Open wells and tanks.—Three tubes each of four dilutions 10 c.c., 1 c.c., 0.1 c.c. and 0.01 c.c. As the volume of work was large, 3 tubes were chosen instead of 5 to save incubator space. It was also considered that the accuracy of (m.p.n.) would not be very much affected thereby. Chemical samples were examined for the following items: colour, odour, turbidity, pH, total solids, electrical conductivity, hardness (carbonate and non-carbonate), iron, acidity, different forms of nitrogen and dissolved oxygen. Procedures as outlined in standard methods of American Public Health Association (eighth edition) were strictly adhered to in all cases.

Methods of statistical analysis.—*Bacteriological results:* An adequate description of the behaviour of each well over a length of time is the first step in the statistical analysis. The bacteriological results show that the same well sometimes has low probable numbers, while at other times high probable numbers are met with. Occasional external contamination, which is almost inevitable under village conditions, introduces a further complication. A method of description which summarizes the range of variability of the probable numbers without undue emphasis on high probable numbers should, therefore, be useful.

The method selected is to describe the well by the proportion of examinations when it gave an estimated probable number of *coliforms* of (a) over 24 per 100 c.c., (b) under 4.5 per 100 c.c., and (c) between 4.5 and 24 per 100 c.c.

If the true density is 10 per 100 c.c., the chances of getting (a) and (b) are respectively 0.071 and 0.054 for the dilutions used in the bacteriological examination. Water giving result (a) could, therefore, be presumed to have a true density greater than 10 per 100 c.c., (b) true density less than 10 per 100 c.c., and (c) true density of 10 per 100 c.c.

In comparing two groups of wells of different categories it was necessary to assume heterogeneity between wells in the same group. The above method of description was suitable for such analysis. Each well was rated according to the proportion of examinations when it gave an estimated number of over 24 per 100 c.c. and the problem of comparison between groups reduced itself to a comparison of the average values of the proportions in the two groups. There are theoretical objections to a direct comparison of the mean values of proportions. To overcome these objections the proportions were converted into degrees by the inverse Sine Transformation $p = \sin^2 \theta$ (Bartlett, 1936) and a straightforward analysis of variance was carried out on the transformed variable. This analysis, therefore, compared the groups of wells according to the frequency with which they gave estimated probable number of over 24 per 100 c.c.

A similar analysis was also carried out by considering the proportion of examinations when the wells gave probable numbers less than 4.5 per c.c.,

Chemical results.—Analysis of variance technique is used in interpretation of chemical data.

Character of soil experimental area.—Representative soil samples from different layers were collected from borings made in the experimental area for sinking new tube-wells. The location of the boring is indicated in the Map (see page 213). The soil samples were analysed for their effective size and uniformity coefficient and the results are summarized in Graph 1.

There is marked heterogeneity in the make-up of soil in top layers but the water-bearing sands from which wells draw water are somewhat similar. The effective size of sand in this layer ranged from 0.15 mm. to 0.20 mm. and uniformity coefficient from 1.5 to 2.8.

Ground-water and meteorological conditions.—The investigations described in the present paper were carried out from beginning of April 1944 to the end of June 1946. Throughout this period systematic observations on rainfall, ground-water

SECTION OF SOIL IN EXPERIMENTAL AREA (FROM BORINGS)

SCALE—25' = 1".

PERVIOUS SAND

E.S.

U.C.

SAND & SILT

0.01 to 0.1

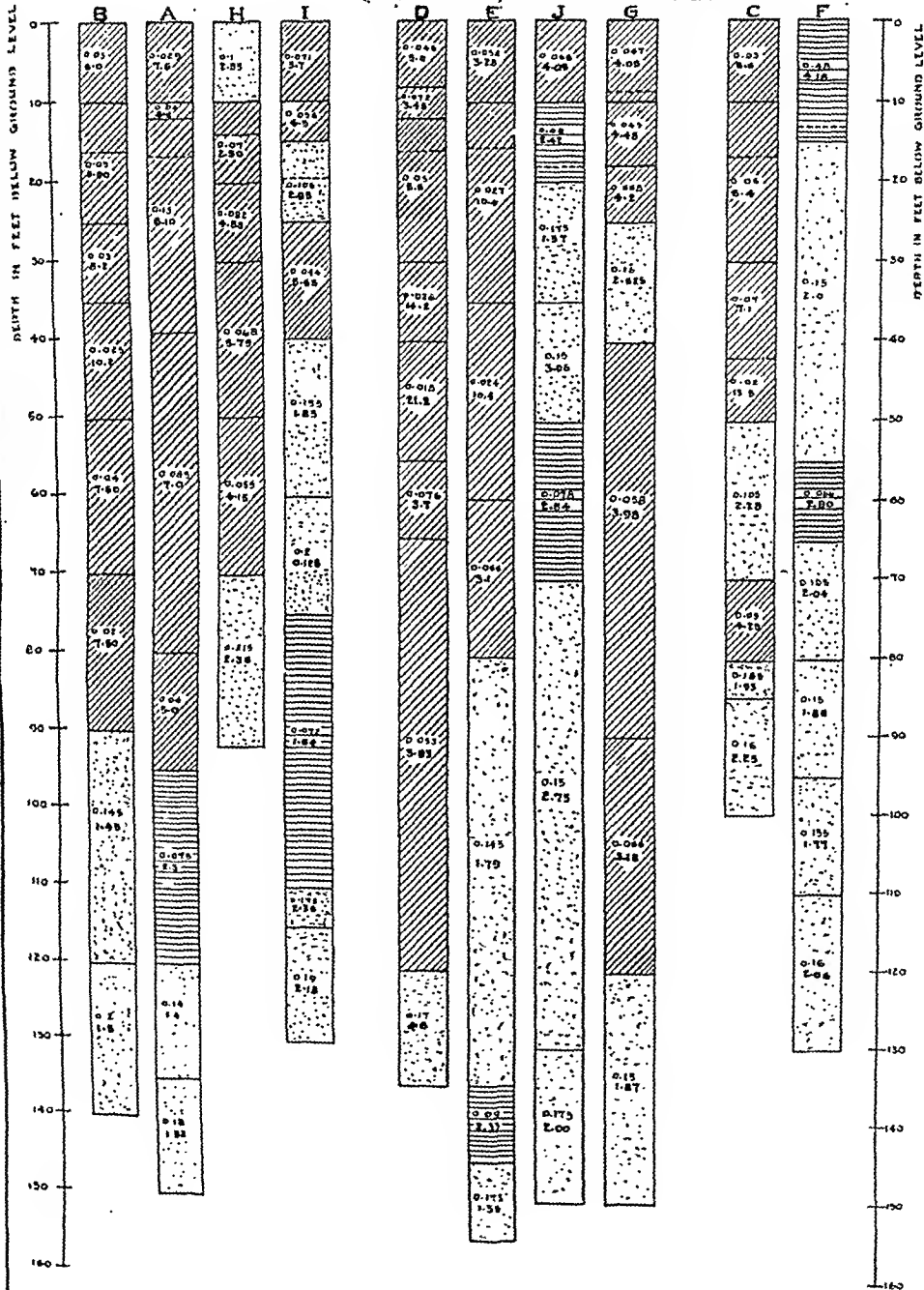
< 30

SAND SILT & CLAY

< 0.1

> 30

--- RENGES --- WATER TABLE
E.S. --- EFFECTIVE SIZE
U.C. (ANTIQUE FIGURES) --- UNIFORMITY COEFFICIENT



(A to J DENOTE THE SAMPLING POINTS INDICATED IN THE MAP)

table, air and water temperature and humidity were made and the results are presented in Graph 2.

Based on this data the period of study has been divided into seasons as indicated in Graph 2.

Part III.

TUBE-WELLS.

The 134 tube-wells selected for sampling were observed once a fortnight for bacteriological examination and once every three months for chemical examination for 2 years and the following observations are based on this data.

Effect of sanitary protection and location of wells on bacteriological quality of water obtained from them.—The bacteriological quality of water as revealed by presumptive *coliform* count of water samples collected from wells with different grades of sanitary protection are summarized in Tables II and III :—

TABLE II.

Percentage of observations when the estimated probable number is less than 5 (m.p.n. per 100 c.c.).

Area.	Private or public.	Grade of sanitary protection.	1ST SEASON.		2ND SEASON.		3RD SEASON.		4TH SEASON.	
			Number of wells.	Percentage.	Number of wells.	Percentage.	Number of wells.	Percentage.	Number of wells.	Percentage.
Singur ...	Private ...	A	19	84.1	18	69.8	15	85.1	16	92.2
		B	9	80.3	8	51.8	8	49.2	9	86.3
	Public ...	A _p	12	82.9	12	58.5	11	92.7	11	93.5
		B _p	26	66.2	26	66.8	26	90.7	26	78.6
		C _p	12	66.5	12	68.0	11	86.0	12	88.1
Balarambati ...	Private ...	A	20	55.6	10	61.0	9	97.6	8	94.9
		B	9	93.2	9	60.8	9	92.2	8	86.3
		C	3	88.6	3	71.0	3	88.6	3	88.6
	Public ...	A _p	7	77.6	7	77.4	6	84.9	7	86.1
		B _p	16	60.8	16	58.2	14	78.7	16	77.0
		C _p	3	75.1	3	26.2	3	79.9	2	93.3

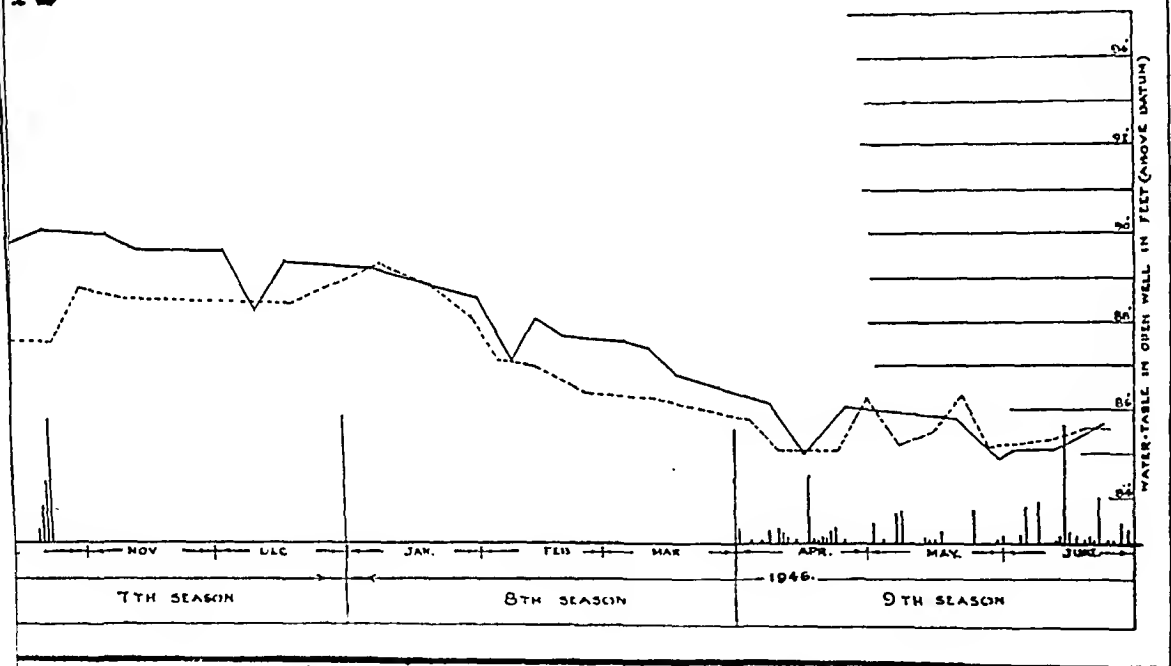


TABLE III.

Percentage of observations when the estimated probable number is more than 24 (m.p.n. per 100 c.c.).

Area.	Private or public.	Grade of sanitary protection.	1ST SEASON.		2ND SEASON.		3RD SEASON.		4TH SEASON.	
			Number of wells.	Percentage.	Number of wells.	Percentage.	Number of wells.	Percentage.	Number of wells.	Percentage.
Singur ...	Private ...	A	19	4.7	18	11.0	15	3.1	16	2.3
		B	9	1.7	8	16.4	8	8.2	9	2.8
	Public ...	A _p	12	4.7	12	10.4	11	1.3	11	0.0
		B _p	26	9.4	26	11.3	26	2.8	26	4.6
		C _p	12	14.9	12	7.8	11	3.5	12	2.4
Balarambati ...	Private ...	A	10	15.1	10	12.7	9	0.0	8	0.6
		B	9	3.3	9	4.8	9	7.8	8	1.6
		C	3	0.0	3	1.7	3	11.4	3	0.0
	Public ...	A _p	7	5.5	7	0.0	6	4.8	7	5.4
		B _p	16	20.9	16	19.9	14	9.1	16	7.4
		C _p	3	15.0	3	41.5	3	4.1	2	0.0

Under soil conditions and depths obtaining in the area under observation the results presented above show that the differences in the degree of sanitary protection do not have any marked effect on the bacteriological quality of water. Generally, the quality of water in public tube-wells is as good as in private tube-wells. Zonal differences are also not significant and there is very little difference in the overall bacteriological picture of wells located in different areas or villages.

Effect of seasons on bacteriological quality of water.—The bacteriological quality of water obtaining in tube-wells during the different periods are summarized in Table IV :—

TABLE IV.

Percentage of observations when the estimated probable number is <5 and >24 (m.p.n. per 100 c.c.).

Season.	SINGUR.			BALARAMBATI.		
	Number of wells.	Percentage observation.		Number of wells.	Percentage observation.	
		<5	>24		<5	>24
1st season ... (2nd April, 1944 to 19th June, 1944).	78	75.3	7.0	48	72	11.0
2nd season ... (20th June, 1944 to 1st October, 1944).	76	65.0	11.0	48	61.0	10.0
3rd season ... (2nd October, 1944 to 25th December, 1944).	71	88.3	3.2	44	88.0	5.0
4th season ... (26th December, 1944 to 1st April, 1945).	74	86.8	2.6	44	86.0	3.0
5th season ... (June to September 1945).	19	74.3	6.4	6	61.6	12.4
6th season ... (October to December 1945).	32	91.8	1.4	22	89.8	1.7
7th season ... (January to March 1946).	32	87.8	3.7	22	94.9	0.5
8th season ... (April to June 1946)	31	81.0	5.4	22	82.0	1.8

The general effect is marked in 1st year monsoon months (2nd season) when the numbers recorded from wells showed significant increase. The effect was not so marked in the 2nd year.

Effect of depth of well on bacteriological quality.—The tube-wells that are sampled represented a wide range of depths varying from 50 ft. to 250 ft. and the

effect of increase of depths of tube-wells on bacteriological quality was, therefore, studied. Some of the results are summarized in Table V. The correlation between depth of wells and the bacteriological quality of water in the 2nd season is presented in Table VI.

TABLE V.

Percentage of observations when m.p.n. of coliform exceeded 24 per 100 c.c.

Depth.		Number of wells.	1st season.	2nd season.	3rd season.	4th season.
D. 75 ft. to 100 ft.	...	35	8	11	3	3
D. 101 ft. to 125 ft.	...	42	10	11	4	3
D. 126 ft. to 150 ft.	...	16	7	3	2	4
D. 151 ft. to 200 ft.	...	7	1	9	4	2

TABLE VI.

Correlation between depth of wells and proportion of samples with estimated probable number of gas-formers more than 24 per 100 c.c.

Groups.				Number of wells.	Correlation coefficient.	Significance.
Singur	...	{	Private	21	0.1706	Not significant.
			Public	47	0.0283	„
Balarambati	...	{	Private	10	0.1504	„
			Public	13	0.0965	„
TOTAL				91	0.0817	„

The results show no significant difference in the bacteriological quality of water obtained from different depths; this is in conformity with the earlier findings that water of high bacteriological purity is obtainable even at shallow depths in these areas and further increase in depth does not appreciably affect the bacteriological quality. It would appear from these findings that wells need not be deeper than

50 ft. (which is the least depth of the wells under observation) from the point of view of safety of water obtained. This depth is not necessary for ensuring protection against sub-surface contamination; but it is the minimum depth required in this area for placing the strainer in a suitable stratum of sand and ensuring a yield sufficient for domestic use.

Electrical conductivity, pH and temperature of the bacteriological samples.—It was considered that sharp changes in pH, temperature and electrical conductivity of the water samples collected from a well from time to time might indicate the presence or possibility of any strong underground steam of pollution flowing into the sampling wells. According to Dyer *et al.* (*loc. cit.*) electrical conductivity is a highly sensitive chemical test of underground flow of pollution. All the bacteriological samples were, therefore, examined for these items.

The temperature of the samples showed very little variation.

Mean pH values of some of the wells through the different seasons are presented in Table VII :—

TABLE VII.

Mean pH value.

Well number.	1st season.	2nd season.	3rd season.	4th season.
22	7.3	7.4	7.2	7.3
16	7.1	7.2	7.2	7.1
152	7.2	7.3	7.2	7.3
113	7.2	7.2	7.4	7.2
34	7.3	7.5	7.4	7.4
130	7.3	7.3	7.3	7.3
46	7.3	7.4	7.3	7.3
7	7.2	7.3	7.3	7.2
1	7.2	7.3	7.3	7.2
112	7.3	7.3	7.2	7.2
46	7.2	7.3	7.2	7.2
125	7.3	7.3	7.3	7.3

The pH values showed too little variation for any statistical analysis.

The electrical conductivity data of a few of the wells are summarized in Table VIII :—

TABLE VIII.
Variation in electrical conductivity of a number of wells at selected depths. (C. V. denotes coefficient of variation.)

Well number.	Depth in ft.	1ST SEASON.		2ND SEASON.		3RD SEASON.		4TH SEASON.		5TH SEASON.		6TH SEASON.		7TH SEASON.		8TH SEASON.		9TH SEASON.	
		Mean.	C. V.	Mean.	C. V.	Mean.	C. V.	Mean.	C. V.	Mean.	C. V.	Mean.	C. V.	Mean.	C. V.	Mean.	C. V.	Mean.	C. V.
S28	89	535	5.9	494	6.2	505	4.7	592	5.0
S16	75	519	2.1	534	2.8	526	2.8	558	4.5	551	1.9	550	Nil	592	8.7	600	Nil	540	9.1
S152	100	508	6.7	571	6.4	578	3.3	581	3.1
S113	124.5	684	2.9	661	2.8	664	3.9	674	4.6
S34	148.6	464	1.9	435	6.3	455	1.1	471	3.3
S150	173	585	3.2	572	7.3	577	3.0	596	0.9
S16	222.3	501	4.5	501	8.5	489	5.8	501	1.7
B37	80	575	4.5	566	3.6	572	2.3	566	5.8
B1	100	606	4.9	609	1.7	600	4.4	642	2.0	629	7.0	631	1.7	630	4.3	602	3.8
B112	125	566	6.0	548	5.2	563	4.6	575	Nil	569	2.2	552	5.6	585	1.7	581	6.5
B46	182	551	3.0	550	7.1	538	4.2	555	3.3	542	8.4	565	2.4	570	Nil	550	Nil.
B125	200	601	1.7	594	8.3	598	2.5	600	Nil	616	1.1	595	1.5	597	0.8	600	Nil.

In the earlier work carried out by Dyer *et al.* (*loc. cit.*) it was observed that the coefficient of variation in conductivity determination due to experimental errors involved in routine sampling and analysis was of the order of 8.5 units. Taking 8.5 as the possible variation, the data presented in the table do not reveal variations in the conductivity of the bacteriological sample sufficient to indicate underground streams of pollution flowing into wells. Similarly, analysis of the data for all the other wells observed in this investigation also showed no indication of underground pollution.

Average bacteriological quality of tube-well waters obtaining under rural conditions.—Except for the seasonal effect, the bacteriological quality of water obtained from different tube-wells was more or less the same. The data for all the tube-wells have, therefore, been pooled together and the average quality of the water in the different seasons is presented in Table IX:—

TABLE IX.

Average bacteriological quality of tube-well waters as shown by the frequency (as percentage of total number of samples examined) of samples in their specified ranges.

Season number.	Number of wells.	M.P.N. OF GAS-FORMERS IN 100 C.C.				TOTAL AGAR COUNT PER C.C.				
		Total number of observation.	5	5-24	24	Total number of observation.	0-20	20-50	50-100	100
1	134	833	69.0	16.2	14.8	722	68.6	19.2	6.2	6.2
2	134	814	62.5	20.4	17.1	527	47.2	24.3	12.2	16.3
3	134	588	79.0	9.9	10.2	315	61.9	17.8	8.5	11.8
4	134	709	80.1	11.9	8.0	207	62.8	15.0	10.1	12.1
5	25	129	58.1	23.3	18.6
6	54	354	74.9	14.4	10.7
7	54	383	81.2	11.2	7.6
8	53	337	73.9	15.4	10.7

The examination of 3,566 samples collected from 134 tube-wells during a period of 2 years showed that in not more than 20 per cent observations the water showed a true *coliform* density greater than 10 per 100 c.c. A total agar count of over 100 per c.c. generally corresponds to an estimated m.p.n. of *coliform* of over 24 per 100 c.c. (or a true probable m.p.n. of over 10). A true probable density of less than 10 appears to correspond to an agar count of less than 20. The total agar count was not more than 100 in 90 per cent samples.

Nature of gas-formers in samples of water from tube-well when the numbers exceeded 10 per 100 c.c.—When the 24 hours' count of gas-formers recovered from the tube-well samples exceeded 10 per 100 c.c., one of the positive tubes was plated on MacConkey agar and 2 typical colonies chosen from the plate were characterized

by 'IMVIC' reactions. The results of these studies are summarized in Table X₁:—

TABLE X₁.

Nature of gas-formers isolated from tube-well samples.

Total number of samples examined	2,600
Number of samples plated	387 (15%)
Instances when m.p.n. of gas-formers were less than 10 per 100 c.c.	2,213 (85%)

Type of organism.				As percentage of total number of samples examined.
<i>B. coli</i> I	...	++	--	2.4
<i>B. coli</i> II	...	—+	---	
<i>I.A.C. intermediates</i>	11.6
<i>aerogenes</i>	
<i>cloacæ</i>	
Irregulars	1.0

Origin of the high numbers of gas-formers in tube-wells.—During the earlier part of the investigation it was observed that in a few tube-wells high numbers of gas-formers were repeatedly recovered and experiments were, therefore, carried out to determine the origin of these high numbers. Ten wells were investigated in this connection; in eight out of the 10 wells the high numbers were traced to external contamination getting into the pump. This contamination lingered on the leather bucket or organic débris inadvertently put into the pumps and contributed the high numbers off and on. A small piece of coco-nut shell covered with vegetable débris was discovered inside one of the pumps. In one of the wells it was observed that the sand that came up along with the water contained high number of gas-formers. When the buckets were replaced and well pumps otherwise properly disinfected the wells showed normal behaviour.

Viability of the gas-formers on the contaminated materials collected from the tube-well pumps.—A few of the contaminated materials collected from tube-wells were kept in a small quantity (25 c.c. to 50 c.c.) of sterile water in the laboratory in order to determine the viability of the organisms present in them. These materials were sampled periodically for determination of the m.p.n. of gas-formers present in them. The procedure adopted for this purpose was as follows: The leather buckets or other materials were shaken up with 300 c.c. of sterile water and the

samples of water thus obtained were inoculated into MacConkey broth tubes. The results of these experiments are summarized in Table XI :—

TABLE XI.

Well number.	Nature of material studied.	Technique.	At the end of time in days.	Mp.n. of gas-formers in 100 c.c. suspension.	Nature of organisms.
BS1	Leather bucket	Kept in sealed Petri-dish covers containing sterile tube-well water.	0	12,247	I.A.C. group of organism.
			22	+	
			52	+	
			103	140	
			166	58	
			230	<58	
			310	<58	
B94	" "	" "	0	+	I.A.C. group.
			19	1,807	
			47	24	
			99	196	
			162	37	
			226	2	
			306	2	
S117	Sand ...	Kept in 100 c.c. sterile water in a 150 c.c. flask.	0	820	I.A.C. group.
			19	126	
			48	—	
			103	—	
BS1	Coco-nut shell	Kept in sealed Petri-dish covers containing sterile tube-well water.	0	3,844	I.A.C. group.
			21	148	
			52	—	
			103	—	

+ represents all tubes positive (>12,247).

— represents absent in 50 c.c.

The results show that the organisms can thrive in leather buckets over very considerable periods and that in the case of coco-nut shell and sand that the period of their viability is much shorter. The chances of external contamination getting into the pump, especially the leather buckets, should be avoided if the occasional high bacterial numbers in tube-well samples are to be obviated. The use of contaminated water for priming the pumps and use of bad quality leather are the main sources from which the leather buckets can derive lactose-fermenters in the tube-well even though the subsoil water is pure.

Effect of using contaminated water for priming tube-well pumps.—The cheap hand pumps with open taps generally used in tube-wells in these areas often require the use of contaminated water from nearby ponds or other sources for priming.

A few experiments were carried out in order to see the effect of this procedure on the bacteriological quality of water drawn from tube-wells. The results are summarized in Table XII :—

TABLE XII.

Effect of priming tube-well pumps with contaminated water on m.p.n. of gas-formers recovered from them.

Well number.	M.p.n. of gas-formers in 100 c.c. of water samples used for priming.	M.p.n. of gas-formers (per 100 c.c.) in samples collected after pumping out varying amounts of water.							
		Gallons of water pumped out.	1	2	3	4	5
S108	>4,500	Gallons of water pumped out.	1	2	3	4	5
		Number of gas-formers in 100 c.c.	—	24	2	5	9
S102	>4,500	Gallons of water pumped out.	1	2	3	4
		Number of gas-formers in 100 c.c.	37	24	24	58
S142	4,500	Gallons of water pumped out.	1	2	3	4
		Number of gas-formers in 100 c.c.	—	5	7
S74	250	Gallons of water pumped out.	1	2	3	4
		Number of gas-formers in 100 c.c.	140	37	58	24
S101	>4,500	Gallons of water pumped out.	2	4	6	10	14	18	22
		Number of gas-formers in 100 c.c.	24	9	9	24	24	5	12

Results are rather erratic but nevertheless indicate that the priming water introduces a comparatively small amount of pollution and the extent to which this

will persist in the water samples will depend upon the condition of the pump. Generally, the contamination introduced along with priming water is pumped out with the first few gallons.

Persistence of high coliform numbers.—For this purpose the wells that showed more than ten *coliforms* after twenty-four hours' incubation of tube were observed every third day until one m.p.n. of less than ten was recorded. Table XIII shows the correlation between two consecutive observations:—

TABLE XIII.

Persistence of high number of coli ærogenes in tube-wells—number of instances when the stated m.p.n. were recovered.

M.p.n. of <i>coliforms</i> in 2nd observation.	1st observation—m.p.n. of <i>coliforms</i> in 100 c.c.													
	—	2	5	9	12	17	24	37	58	70	149	196	312	Total.
<2 (Absent in 50 c.c.)	1	1	...	1	1	3	4	3	1	1	16
2	1	2	1	4
5	2	...	1	
9	2	2
12	—
24	1	...	1	2
37	—
58	—
70	1	1
149	—
196	—
312	—
TOTAL ...	1	1	...	3	1	7	6	4	1	2	2	28

We have adopted 24 as the upper limit of actual m.p.n. for the configuration of tubes used corresponding to a true m.p.n. of 10. Out of fifteen instances with m.p.n. 24 or more in only one case the m.p.n. in the consecutive observation was also greater than 24. In nine cases the successive m.p.n. recorded was less than two and in three cases it was two. In three out of fifteen or in only 20 per cent cases there appears to be a need to bring down the high numbers by disinfection.

Effect of frequent usage and heavy demand on bacteriological quality of water.—Four wells—three being used heavily and frequently and one comparatively little used—were observed all through the year to see what effect the heavy demand had on the quality of water and how they compared with the general run of wells and with those that were little used. These were not chlorinated after repairs. One point that stands out prominently is that the heavily-used wells go out of order very frequently and that high numbers make their appearance immediately after effecting repairs. Apart from this, they compare quite favourably with the other wells.

TABLE XIV.

S71—well in the evacuation camp, rarely used.

S73, S74 and S4—market tube-wells, heavily and frequently used.

M.p.n. of organism in 100 c.c.	Number of times the well showed the stated m.p.n.				
	Well number.	Jan.-March 1945.	April-June 1945.	July-Sept. 1945.	Oct.-Dec. 1945.
4.5	S71	9	9	7	6
	S73	9	8	10	10
	S74	6	5	6	4
	S4	5	7	11	7
4.5 to 24	S71	...	1	1	...
	S73	...	1	1	1
	S74	1	3	...	3
	S4	...	2	...	1
24	S71
	S73	...	1
	S74	1	1	1	1
	S4	2	1	1	1

TABLE XV.

Comparing the three much-used wells with the general run of wells.

M.p.n. in 100 c.c.	Percentage of observations when the wells gave stated m.p.n. of coliforms.							
	Jan. to March. <4.5 >24		April to June. <4.5 >24		July to Sept. <4.5 >24		Oct. to Dec. <4.5 >24	
Much used ...	83.3%	12.5%	69.0%	10.3%	90.0%	6.6%	75.0%	7.1%
Rest ...	96.8%	1.6%	76.6%	10.6%	90.0%	5.0%

	Total number of observations.	Percentage of observations when wells gave m.p.n.		
		<4.5	<4.5 >24	>24
		Per cent.	Per cent.	Per cent.
Much used Nos. S73, S74 and S4.	65	83.07	10.76	6.15
Private S105, S92 and S148.	39	92.3	2.56	5.12

Effect of chlorination of tube-wells on the bacteriological quality of water.—Thirty-nine tube-wells were divided into 3 equal categories as follows: A—in which the wells were disinfected once every three months and after repair, B—in which the wells were disinfected only after repair, and C—which were not disinfected at all. The following procedure was used in carrying out disinfection: The pump was disconnected and the 50 p.p.m. chlorine solution poured into the tube-well in quantities equivalent to the capacity of the tube. The pump was also immersed in the chlorine solution. After standing overnight the pump was re-fitted and all the chlorine solution pumped before sampling.

The wells were observed once a month for the first 3 months, the first observation after chlorination being done after about a week. In this period only two wells belonging to the B category were repaired and chlorinated. After September the design was modified to allow for possible variation in the behaviour of wells in different zones and they were also observed more frequently—every fortnight. Six wells from Singur and six from Balarambati were selected for each of the three categories. Three wells belonging to A from Singur and three from Balarambati were chlorinated during the same week and the other six wells during the next week. The first observations were taken one week after chlorination. In this interval only one A well was repaired and disinfected, but nine in B.

The results are presented in Table XVI :—

TABLE XVI.
Effect of periodic disinfection of wells.

M.p.n. of coliforms per 100 c.c.	NUMBER OF INSTANCES WHEN THE STATED M.P.N. WAS RECOVERED IN THE DIFFERENT CATEGORIES OF WELLS.					
	First half.			Second half.		
	A	B	C	A	B	C
<4.5	36	43	31	63	60	82
<4.5 >24	2	4	3	...	1	1
>24	7	5	2	2	2	3

These results reveal that there is no significant difference among the three categories and that regular chlorination once in three months does not prevent the occasional occurrence of high numbers. As only two wells belonging to the B category were repaired in the first half and none in the second half, there is practically no difference between the B and C groups of wells and the experiment has not provided any evidence showing the need for chlorination after repair.

Having known that chlorination at three-monthly intervals does not prevent the occurrence of high numbers, the next point of interest was the immediate effect of chlorination as shown by the first observation one week after chlorination in each of the three categories.

TABLE XVII.
Persistence of the effect of disinfection with chlorine in tube-wells as observed in first samples of water taken a week later.

M.p.n. of coliforms per 100 c.c.	NUMBER OF INSTANCES WHEN THE STATED M.P.N. WAS RECOVERED IN THE DIFFERENT CATEGORIES OF WELLS.					
	First half.			Second half.		
	A	B	C (Not disin- fected.)	A	B	C (Not disin- fected.)
<4.5	11	8	10	9	11	9
<4.5 >24	...	4	1	3	...	2
>24	1	1	2	1	...	1

These figures too are not significant and the effect of chlorination does not persist even up to a week.

In light of the above data further experiments were carried out to determine the effect of chlorination on the quality of water obtained in tube-wells. Thirty-six wells were divided equally into three classes A, B and C—A being disinfected regularly once in three months, B only when repaired and C never disinfected at all. Six wells of each category were chosen from Singur and six from Balarambati. Disinfection was carried out as in the previous experiments. The disinfected wells along with B and C wells belonging to the particular area were observed a week after disinfection and every fifteenth day thereafter. The Singur A wells were disinfected on 18th December, 1945 and 27th December, 1945 in the first quarter and on 28th March, 1946 and 2nd April, 1946 in the second quarter. At Balarambati the A wells were disinfected on 22nd December, 1945, 28th December, 1945, 29th March, 1946 and 3rd April, 1946. In the first quarter A wells were repaired thrice. B and C were not repaired at all, in the second A was repaired thrice and B seven times. The results of these experiments are presented in Tables XVIII and XIX :—

TABLE XVIII.

Disinfection of tube-wells.

M.p.n. of coliforms in 100 c.c.	NUMBER OF INSTANCES WHEN THE STATED M.P.N. WAS RECOVERED IN THE DIFFERENT CATEGORY OF WELLS.					
	First quarter.			Second quarter.		
	A	B	C	A	B	C
<4.5	65	67	70	60	57	61
<4.5 >24	7	5	5	9	10	7
>24	2	4	...	1	3	2

TABLE XIX.

Persistence of effect of disinfection of tube-wells.

M.p.n. of coliforms in 100 c.c.	NUMBER OF INSTANCES WHEN THE STATED M.P.N. WAS RECOVERED IN THE DIFFERENT CATEGORY OF WELLS.					
	First quarter.			Second quarter.		
	A	B	C	A	B	C
<4.5	11	12	11	9	7	9
<4.5 >24	1	3	2	1
>24	1	2	2

TABLE XXI.

Chemical quality of samples from tube-wells in different depth.

Seasons.	Carbonate hardness (p.p.m. CaCO ₃).						Total solids (p.p.m.).					
	D1		D2		D3		D1		D2		D3	
	N.	M.	N.	M.	N.	M.	N.	M.	N.	M.	N.	M.
April to June 1945 ...	27	270	32	277	15	291	25	355	32	334	15	369
July to September 1945 ...	14	303	16	272	7	337	16	330	15	342	9	333
October to December 1945 ...	9	311	8	308	9	342	9	270	7	230	8	348
January to March 1946 ...	10	245	16	287	6	276	10	330	14	321	7	304
April to June 1946 ...	16	351	12	354	7	372	17	427	14	426	7	442

Seasons.	Chlorides (p.p.m. Cl).						Total iron (as p.p.m. Fe).					
	D1		D2		D3		D1		D2		D3	
	N.	M.	N.	M.	N.	M.	N.	M.	N.	M.	N.	M.
April to June 1945 ...	28	19.4	36	18.7	16	22.6	27	0.91	34	1.08	16	0.43
July to September 1945 ...	18	20.1	18	17.9	11	16.8	18	2.01	16	2.00	11	2.35
October to December 1945 ...	12	22.1	7	16.0	7	16.1	13	1.61	9	1.53	8	1.37
January to March 1946 ...	12	20.8	15	16.3	8	16.1	12	1.48	13	1.41	8	2.28
April to June 1946 ...	16	19.8	14	18.4	8	15.0	16	1.55	15	2.54	8	1.18

N indicates number of observations.

M = mean value.

D1 = 60 ft. to 100 ft.

D2 = 101 ft. to 125 ft.

D3 = 126 ft. to 150 ft.

TABLE XXII.

Average chemical quality of samples collected from tube-wells.

Chemical characters.	Seasons.							
	1st.		2nd.		3rd.		4th.	
	N.	M.	N.	M.	N.	M.	N.	M.
Carbonate hardness (as p.p.m. CaCO ₃)	117	304	115	340	112	299	109	291
Total solids (p.p.m.)	113	435	107	434	111	351	106	343
Chlorides (as p.p.m. Cl)	120	17	107	18	112	18	110	17
Iron (as p.p.m. Fe)	116	1.72	107	1.81	106	1.56	100	1.75

TABLE XXII—concl'd.

Chemical characters.		Seasons.									
		5th.		6th.		7th.		8th.		9th.	
		N.	M.	N.	M.	N.	M.	N.	M.	N.	M.
Carbonate hardness (as p.p.m. CaCO_3).	(as	74	279	37	304	26	320	32	266	35	359
Total solids (p.p.m.)	...	72	353	40	335	24	283	31	348	38	432
Chlorides (as p.p.m. Cl)	...	80	20	47	18	26	18	35	18	38	18
Iron (as p.p.m. Fe)	...	77	0.81	45	2.12	30	1.50	33	1.72	39	1.76

the public tube-wells and 94 per cent of private tube-wells were in some sort of working order. Forty-three per cent of the public tube-wells were in poor repair, as against 21 per cent of private tube-wells. The maintenance of public tube-wells is of a definitely lower standard than that of private tube-wells.

The defects noticed in tube-wells were the following :—

1. Broken studs and bolts.
2. Worn out leather buckets.
3. Leaky leather washer and valve at foot of pump.
4. Pump not functioning.
5. Filter choked.
6. Head of pump broken.
7. Pump handle broken.

The cement plug at the base of the pump was omitted or broken in 41 per cent of public tube-wells and 84 per cent of private tube-wells.

The wells yielding less than 80 gallons per hour were unpopular and not in use. A few tube-wells giving red water or brackish water were also not in use.

From the study of the data, it appears that the 221 tube-wells in Singur and Balarambati had to be repaired 329 times in 16 months or 20.56 times a month with a probable error of ± 8.8 . Each tube-well requires attention on an average once in 10.75 months, the actual time varying probably from 7.53 to 18.8 months.

The public tube-wells numbering 156 were repaired 297 times, in 16 months, while the private tube-wells numbering 65 were repaired only 32 times in the same period. This corresponds to one repair for a public tube-well once in 8.45 months against a repair for a private tube-well once in 32.5 months. This shows that there is much greater wear and tear and perhaps more irresponsibility about the use of a public tube-well than about a private one. This feature was brought out at the sanitary survey too, when among the public tube-wells, 20 per cent were totally defunct and 43 per cent in poor repair, whereas among private tube-wells only 6 per cent were totally defunct and 21 per cent in poor repair.

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After the institution of a maintenance service 41 per cent of the repairs were effected on receipt of notification from the public and the other 59 per cent were repaired by the crew on their own initiative. The public is, therefore, getting slowly educated to take an interest in the maintenance of the tube-wells. Reports regarding the necessity for a repair were received within 2 days in 51 per cent of the cases and within 10 days in 78·7 per cent of the cases. On receipt of a report, the repairs were taken up within a day in 64·4 per cent of the cases, within 3 days in 89 per cent of cases and within 7 days in 95 per cent of cases. The cost of materials used in repairs was less than 1 rupee in 58 per cent of the cases and less than Rs. 4-8-0 in 83·4 per cent of cases. The average cost of materials used per repair was about Re. 1-10-0. It was about Rc. 1-14-0 per tube-well per annum excluding re-sinking charges. Eighty-five per cent of the repairs were effected by the mistry (single-handed) and without other help.

The repairs required were in the following descending order of frequency :—

				Percentage.
<i>Nature of repair and replacement.</i>				
1.	Replacement of	leather bucket	...	46
2.	"	bolts and nuts for handles	...	35
3.	"	leather valve at base	...	32
4.	"	valve screw	...	16·7
5.	"	piston rod	...	16
6.	Repairs to head	11
7.	Replacement of	handle	...	8·5
8.	"	plunger	...	7·3
9.	"	head	...	4·5
10.	"	screw	...	3·6
11.	"	entire pump	...	1·8
12.	"	base	...	0·3
13.	"	valve weight	...	0·3

According to data maintained by the Singur Health Centre from July 1944 to June 1946, a public tube-well had to be repaired on an average once in 7·25 months, and the cost of a repair was Rs. 2·56 for materials and 2·23 for labour, i.e. Rs. 4·79 per repair. The cost of maintenance of a public tube-well was Rs. 8·44 per annum in 1945-46 and Rs. 8·03 in 1944-45. This may seem somewhat excessive in view of the fact that the time actually taken for repair was less than 1 hour in 95 per cent of cases. Communications being difficult it is found that a supervisor travelling from a central place can only look after 150 to 200 public tube-wells in 2 union boards.

Practicable bacteriological standards for tube-wells in rural areas.—The high bacteriological standard attainable in controlled piped water-supplies is not applicable to small private supplies in rural areas. Although tube-wells can yield water with *coliform* organisms absent in 100 c.c. it is not possible to attain this result in routine bacteriological examinations because of the various factors inherent in maintenance of tube-wells under rural conditions. Bacteriological standards should, therefore, be the best practicable in tube-wells under rural conditions where they are consistent with—

- Proved absence of surface and sub-surface pollution in the tube-wells.
- Absence of water-borne diseases amongst the users traceable to the well-water.

The standard should be based on observation of large numbers of such wells spread over a wide area and over a sufficiently long period to be of general application.

Evidence on (a) has been presented in this paper. This has been based on fortnightly observations of 134 tube-wells spread over an area of 16 square miles and for a period of 2 years. In the course of the last 3 years cases of cholera and dysentery occurring in the area have been investigated, but no instance has occurred to warrant the suspicion that tube-well water was responsible for any of the cases.

The low coefficient of variation in electrical conductivity of the bacteriological samples, collected from each well from time to time, the absence of any significant variation in bacteriological purity of samples drawn from tube-wells under varying conditions of sanitary conservation at the surface and of usage, and the very low dissolved oxygen in chemical samples from tube-wells prove that they tap a water that is practically cut off from surface water and surface pollution. Their general quality, therefore, represents the purest water obtainable naturally. Many cases of gastro-intestinal diseases in the community were investigated, but no evidence was available to show that tube-well water could be suspected as being responsible for any case. Under these conditions, it was observed that of the samples examined at least 75 per cent show less than 5 gas-formers per 100 c.c. and that only in about 20 per cent of the observations the estimated probable number of gas-formers exceeds 24, that in 60 per cent of the samples the total agar count is less than 20 per c.c. and that it exceeds 100 per c.c. only in 11 per cent of the samples.

If other configurations are adopted in place of the configurations used in these investigations, the range corresponding to a probable true m.p.n. of 10 per 100 c.c. may be expressed as follows:—

Configuration of tubes used for the presumptive test.	Estimated probable numbers of <i>coliforms</i> per 100 c.c. corresponding to a true density of 10 organisms per 100 c.c.
50 c.c. 1 tube 10 c.c. } 1 c.c. } 3 tubes each 0.1 c.c. }	4.5—24
10 c.c. ... 5 tubes	5.2—17.9
50 c.c. ... 1 tube 10 c.c. ... 5 tubes	} 5.2—15.6
50 c.c. ... 1 tube 10 c.c. ... 5 tubes 1 c.c. ... 5 tubes	} 5.0—22.0

Not more than 20 per cent of the very large numbers of samples examined gave probable numbers of *coliform* greater than stated estimated upper limits for a true density of 10 *coliforms* per 100 c.c.

However, if a smaller number of samples had been examined, this 20 per cent would have varied and would have corresponded to the following :—

Number of samples examined.	Percentage of samples where the true density (or in corresponding estimated number) should not exceed 10 <i>coliforms</i> per 100 c.c.
10	40
25	32
50	30
100	27
>100	20

The total agar count at 37°C. may be used as a confirmatory test for natural ground-water and will not exceed 100 per c.c. in 90 per cent of the samples.

The foregoing may be considered as the basis of attainable bacteriological standards applicable for a satisfactory water from tube-wells in rural areas. These standards may be unattainable when the soil conditions differ, and the chances of subsoil contamination are greater than were found in our experimental area.

A number of tube-wells in an area may be looked upon as different points on the distribution system of a homogeneous source of ground-water. These standards may be applicable to the samples from a number of tube-wells in a particular zone, or ground-water stream or ground-water basin to judge the safety of a rural water-supply to a community. Similarly, the standard may be applied to samples collected at different times from one or more sources to judge whether the maintenance of rural tube-wells is satisfactory or not.

The procedure for applying this standard has to be considered in relation to the limited facilities available and intervals between bacteriological examination of rural tube-wells. Although the results on disinfection showed that there is no need for periodic disinfection, it may be a useful practice to disinfect the wells soon after repair and this would greatly minimize the occasional false high numbers. The results on persistence of the occasional high numbers also showed that if a well which shows a high number is sampled 3 or 4 times on consecutive days the numbers are reduced in a short time in all cases except where there may be underground pollution flowing into the wells. It may be possible to impose the standard above detailed provided these two precautions are taken, viz. disinfection of the well soon after the repair, and repeated (3 to 4 times) bacteriological examination of samples on consecutive days when the well shows high numbers.

Part IV.

OPEN WELLS AND TANKS.

Bacteriological quality of tank and open-well water under existing conditions.—The open shallow wells and tanks which are used as drinking water-sources in the area were sampled once in a week for bacteriological and once in 3 months for chemical examination. Bacteriological quality of open well and tank-water under existing conditions is presented in Tables XXIII and XXIV:—

TABLE XXIII.

Nature of source.	Number of wells observed.	MEAN VALUE FOR M.P.N. OF GAS-FORMERS IN 100 C.C. SAMPLE.			
		1st season.	2nd season.	3rd season.	4th season.
Open wells ...	44	4,523	4,850	2,710	1,124*
Tanks	7	3,292	4,532	3,260	2,645

* Based on observation of 12 wells which were left in their existing condition during this season.

TABLE XXIV.

Locality.	Total number of observations.	NUMBER OF TIMES WHEN M.P.N. PER 100 C.C. WERE:—		
		Below 100.	100 to 1,000.	Over 1,000.
Singur ...	256	9	69	178
Balarambati ...	277	5	93	179

There is considerable decrease in the number of bacteria during the 3rd and 4th season, i.e. during the cold months.

Effect of improvements to open wells on bacteriological quality of water.—Under the existing conditions the open wells give extraordinarily high numbers. The wells have to be improved to reduce the numbers considerably before they can be considered for any bacteriological standards.

Seven of the open wells were, therefore, provided with a light cover of sheet-iron and fitted with deep-well-pattern pumps, while seven others were fitted with same pattern of self-priming pumps but no covers; the remaining wells were left in the existing condition to serve as controls. After four months a few of the open wells were provided with cement concrete cover to eliminate chances of any external contamination (Plate IV, fig. 1).

PLATE IV.

Open wells.



FIG. 1.—Open well fitted with pump and concrete cover.

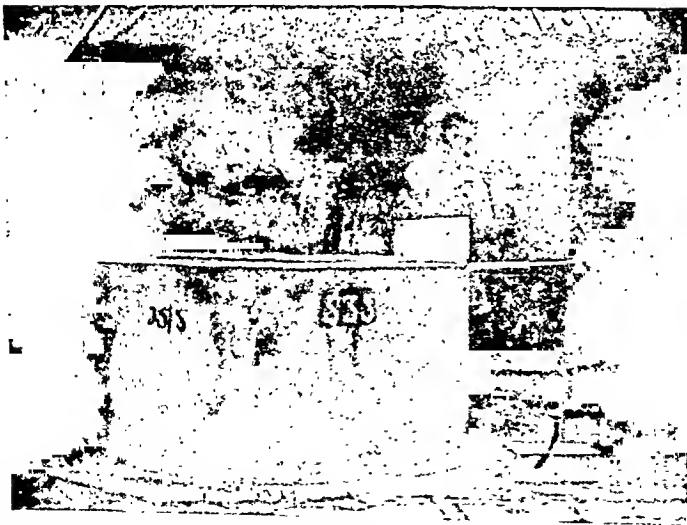


FIG. 2.—Open well fitted with pump only.

TABLE XXV—concl'd.

Kind of well.	Well number.	TRUE PROBABLE DENSITY.						TOTAL.
		LESS THAN 100.		VERY LIKELY 100.		MORE THAN 100.		
		Number.	Per cent.	Number.	Per cent.	Number.	Per cent.	
Unaltered	S54	2	8.30	8	33.30	14	58.30	24
	S30A	2	15.40	5	38.40	6	46.20	13
	S182	0	00.00	5	38.40	8	61.30	13
	B66	4	19.04	9	47.90	8	38.08	21
	B87	6	22.20	9	33.30	12	44.40	27
	B126	3	11.10	10	37.03	14	51.80	27
TOTAL	17	...	46	...	62	...	125
Average of percentage	12.67	...	32.22	...	50.01	...

The results show that the concrete-covered wells with pumps were the best and gave more than 100 *coliforms* per 100 c.c. only in about 22 per cent of the observations. Open wells fitted with pumps also showed great improvement. They are, however, still inferior in bacteriological quality to tube-wells and it has not been possible to obtain the standard of the tube-wells in the existing open wells by the alterations carried out.

Chemical quality of open well and tank water.—Chemical samples collected from open wells and tanks were examined once in three months and results obtained are summarized in Tables XXVI and XXVII :—

TABLE XXVI.

Chemical quality of shallow open-well water.

Chemical characters.	SEASON 1.		SEASON 2.		SEASON 3.		SEASON 4.		SEASON 5.		SEASON 6.		SEASON 7.		SEASON 8.		SEASON 9.	
	SEASON 1.		SEASON 2.		SEASON 3.		SEASON 4.		SEASON 5.		SEASON 6.		SEASON 7.		SEASON 8.		SEASON 9.	
	N.*	Mean.	N.	Mean.	N.	Mean.	N.	Mean.	N.	Mean.	N.	Mean.	N.	Mean.	N.	Mean.	N.	Mean.
Total solids (p.p.m.) ...	34	957.8	41	850.1	24	795.0	20	487.0	14	624.8	17	586.0	20	437.4	25	567.7	26	593.3
Carbonate hardness (p.p.m. CaCO_3).	41	301.0	41	265.1	25	283.8	20	269.6	14	263.1	16	252.6	20	271.3	25	300.8	26	297.7
Chlorides (p.p.m. Cl) ...	40	154.2	42	119.3	25	127.0	20	132.1	14	113.4	19	95.8	20	101.4	25	106.8	26	101.5
Iron (p.p.m. Fe) ...	41	1.01	42	1.77	25	1.14	10	0.73	14	2.4	15	3.0	20	1.9	25	2.8	26	4.0

N* indicates number of wells observed.

TABLE XXVII.

Chemical quality of tank water.

Chemical characters.	SEASON 5.		SEASON 6.		SEASON 7.		SEASON 8.		SEASON 9.	
	N.*	Mean.	N.	Mean.	N.	Mean.	N.	Mean.	N.	Mean.
Total solids ...	5	109.2	6	123.5	6	65.3	6	200.3	6	226.3
Carbonate ...	6	44.0	6	33.3	6	46.2	6	53.0	6	75.0
Chloride ...	6	40.7	6	36.8	6	37.8	6	51.7	6	60.3
Iron ...	5	2.2	4	3.7	6	1.7	6	3.0	6	3.6

N* indicates number of wells observed.

The results show that the chemical quality of shallow open-well water is not as satisfactory as that of tube-wells.

DISCUSSION.

The most important observation arising out of the present studies is that tube-wells of moderate depth can serve as satisfactory sources for safe water-supply in rural areas with soil conditions similar to those at Singur, i.e. over a large part of the Gangetic plain and delta. They yield water that is little affected by comparatively insanitary conditions on the surface, change of seasons, heavy usage or even the use of priming water. The water from the surface is not able to mix directly with the ground-water and the soil gets naturally compacted around the tube-well so as to cut off surface contamination. There is little chance of the ground-water getting contaminated under the prevailing conditions of soil and usage. If a tube-well can yield 250 to 350 gallons per hour, it is appreciated by the community. If its yield falls below 80 gallons per hour, it is abandoned.

Evidence gathered shows that it is practicable to attain a bacteriological standard of not more than 10 *coliforms* per 100 c.c. (within the limits of error applicable to the particular configuration of tubes used for analysis) in 80 per cent of the samples taken with variations applicable to a smaller number of samples, whether from different tube-wells of an area which may be considered homogeneous in hydrological conditions or over a period of time from one or more tube-wells. This standard appears to be consistent with the absence of gastro-intestinal diseases traceable to tube-well water, and may be considered as the standard for adoption in judging the purity of rural water-supplies from tube-wells. If in any place the soil is likely to develop cracks and fissures, the surface water will be able to mix with the ground-water and the samples will naturally fail to come up to this standard.

With the cheap type of pitcher pump commonly used for tube-wells in India an efficient maintenance service is required.

A public tube-well needs on an average 2 repairs in a year. Although one mistry can handle most of the repair jobs single-handed and may require less than an hour for actually carrying out 95 per cent of the repair jobs, he can effectively take charge of not more than 150 to 200 tube-wells owing to difficult condition of travel prevailing in the rural areas. It is desirable to have 2 or 4 mistries under a fitter, working as a team. The local authority or the Government should provide the organization and funds for maintenance, which costs about Rs. 8 per public tube-well per annum.

Whenever a repair is carried out there is likelihood of contamination being introduced and it is desirable to disinfect the tube-well and pump after repair. This may be carried out with a solution of bleaching powder having 50 p.p.m. of chlorine, allowing 12 to 15 hours' contact. The effects of chlorination do not persist for long, and it is not necessary to disinfect a tube-well periodically.

Open shallow wells yield water that is definitely inferior to tube-wells in bacteriological quality. The bulk of the pollution appears to be introduced directly from the surface through the open top, and through buckets and ropes. It appears that very little of the contamination is introduced underground. The soil and the pottery rings and other lining of the open wells are, however, all conducive to the retention of any contamination that may be introduced. When a pump is fitted, there is a considerable improvement in the bacteriological quality of the water. There is a further improvement when the top is closed by a concrete cover and it is practicable to attain a standard of not more than 100 *coliforms* per 100 c.c. in 75 per cent of the samples. This is, however, far inferior to the practicable standard for tube-wells and it is necessary to carry out further investigation on open wells.

The water from open wells had about the same average hardness but contained more chlorides and total solids than the water from tube-wells. This is probably the effect of evaporation and concentration of solids in the unused open wells, which do not appear to be drawing their supply directly from the ground-water, perhaps owing to clay, humus or some other colloidal film on the bottom separating the ground-water from the surface water. The chlorides and total hardness of the tube-well waters were not related to the depth of the strainer, but they changed with the seasons, indicating that there is an underground stream of water changing in quality with the seasons and not directly mixing with the surface water.

Most of the contamination in a well or tube-well takes place through the top. The more accessible and bigger the top, the greater is the contamination.

Tanks are obviously unsuitable as a source of drinking water owing to the unlimited possibilities of contamination. If they are to be used, the water should be disinfected incessantly and supplied through pipes.

SUMMARY.

Investigations were carried out for a period of 2 years (1944-46) on the quality of water drawn from tube-wells and other sources of drinking water, by people in the Singur and Balarambati Union Boards in West Bengal, and on the

problems of maintenance of those sources in a satisfactory condition. These are described.

A preliminary sanitary survey shows that there are 313 sources of drinking water for a population of about 27,000 in an area of 16.5 square miles. Of these 81 per cent, 254 are tube-wells, 55 shallow open wells and 4 tanks. Some tube-wells are privately owned and maintained in a better state of repair than the public ones. All the tube-wells were of 1½ inch diameter and had 12 ft. of strainer. Thirty-two per cent of the tube-wells were in poor repair at the time of the survey and 13 per cent were defunct.

Soil analysis shows that the water-bearing sands in which the tube-well strainers are placed are within a range of effective size from 0.15 mm. to 0.20 mm. and uniformity coefficient varying from 1.5 to 2.8. These sands occur below layers of sand and clay mixed together at depths varying from 50 ft. to 250 ft.

The tube-wells in this area yield water of a quality that is not significantly affected by the degree of sanitary conservation or lack of it at the surface, the mode of usage, depth of strainer, or location of well. It is very slightly affected by change of seasons. The open wells and tanks do not yield waters of such purity, and their quality is more readily changing with the seasons.

The examination of 3,586 bacteriological samples from tube-wells for 2 years shows that the 80 per cent of the samples have a true m.p.n. of not more than 10 *coliform* organisms per 100 c.c.

Seventy-five per cent of the *coliform* organisms recovered from tube-well samples are of the I.A.C. group. *Coliform* organisms are sometimes found lingering for several months on leather buckets of pumps or organic debris or soil that may be introduced into a tube-well. This may account for abnormal *coliform* counts in some cases.

Ordinarily the contamination introduced into a tube-well with impure priming water is pumped out with the first few gallons.

Periodic chlorination of tube-wells does not effect any lasting change in the quality of the water and appears to be unnecessary. It is, however, desirable to chlorinate a pump and tube-well immediately after every repair as there is a likelihood of contamination being introduced during repair.

The samples from tube-wells have very little dissolved oxygen and show very little variation in electrical conductivity.

The water obtained from tube-wells in the area has a somewhat high iron content which is mostly in the ferrous state.

The soil in the area is able to close round the tube-wells compactly in a short time and cut off practically all surface water and surface contamination, except what is unavoidable be introduced through the pump in the way of priming water. Even this does not last long.

The bacterial quality represented by the tube-well samples in this area may be considered to be the highest attainable by uncontaminated ground-water under practical conditions in rural areas allowing for various contingencies.

Although tube-wells give such a good water in the area considered, the pumps attached to them are liable to get out of order. Two hundred and thirty-nine tube-wells had to be repaired 329 times in the course of 16 months. Public tube-wells were used with less consideration and went out of order more frequently than private ones. An efficient maintenance service and arrangements for immediate reporting of defects are essential to get the maximum benefit out of tube-wells. These are not available at present in other rural areas.

The time taken for actual repair does not exceed 1 hour in 95 per cent of the cases and the cost of materials does not exceed Rs. 4-8-0 in 83 per cent of the cases. However, taking the difficulties of road transport into account, one man is required to maintain 150 tube-wells and the total cost of maintenance is about Rs. 2 per tube-well per annum.

Under existing conditions of usage water from open wells and public supplies contain very large numbers of *coliform* organisms. The chances of contamination are too many to consider any practicable bacteriological standard for water to be presumed safe water from such sources. If the open wells are fitted with pumps and covered by concrete slabs, the quality of water improves and can be reduced to less than 100 *coliforms* per 100 c.c. in 75 per cent of samples.

The chemical quality of water from open wells is often inferior to that from tube-wells in this particular area. The hardness is not much less than that of water from tube-wells and the salinity is often more.

The significance of the above findings in evolving practicable bacteriological standards for rural water-supplies is also discussed.

The authors wish to acknowledge with thanks the assistance they received from Mr. B. M. Kapur in carrying out the sanitary survey and maintenance of tube-wells and Mr. G. R. Amritamaharaj who was in charge of sampling and laboratory work in the last 9 months of the inquiry.

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ASCORBIC-ACID REQUIREMENT OF INDIAN ADULT.

BY

H. N. DE,

AND

C. H. CHAKRAVORTY.

(From the Nutrition Research Unit, I.R.F.A., Biochemical Laboratory,
Dacca University, Dacca.)

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INTRODUCTION.

THE requirements of protein, calcium, phosphorus and other minerals for Indian adults have been reported by Basu and his co-workers (Basu and Basak, 1939; Basu, Basak and De, 1941; De and Basu, 1946) from this Laboratory. There is, however, no literature regarding the vitamin requirement of the Indian adults. The study of the requirements of some important vitamins as ascorbic acid, thiamin and riboflavin will, therefore, throw new light on the adult nutrition of India. In consideration of this, the investigation of the saturation requirement of vitamin C of the adult Indian males has been taken up in this Laboratory to see whether it corresponds to that in other countries.

By the method of urinary response to ascorbic acid in test doses, Belser, Hauck and Storvick (1939), Todhunter and Robbins (1940), Lewis, Storvick and Hauck (1943) and Kline and Eheart (1944) found the ascorbic-acid requirement for saturation of adults between 1.0 mg. to 1.6 mg., 1.6 mg. to 1.7 mg., 1.22 mg. to 1.35 mg. and 1.4 mg. to 1.8 mg. per kilogram body-weight, respectively. Storvick and Hauck (1942) by observation of urinary and plasma response to test dose found the value between 1.32 mg. and 2.85 mg. per kilogram body-weight and Kyhos *et al.* (1944) by the plasma-response method observed the ascorbic-acid requirement of an adult in the neighbourhood of 75 mg.

Using the technique of utilization, Purinton and Schuck (1943) determined the requirement of an adult as ranging between 1.50 mg. and 1.86 mg. per kilogram body-weight. Dodds and MacLeod (1944) by studying urinary excretion of three subjects at levels of intake between 82 mg. and 107 mg. found the

saturation requirement varying between 1.23 mg. and 1.60 mg. per kilogram body-weight.

The results obtained by the above workers show that the saturation requirement of ascorbic acid for normal adult except in few cases should lie within the range of 1.0 mg. to 1.86 mg. per kilogram body-weight. It is also observed that there is a wide range of individual variation of the requirement.

EXPERIMENTAL.

The method of urinary response to test dose as modified by Kline and Eheart (*loc. cit.*) has been adopted in the present investigation.

The experiments were conducted on five subjects whose weight ranged from 45 kg. to 53 kg. They were kept under strict observation in the hospital and were served restricted diets containing rice, fish, dhal, vegetable and milk. The average dietary intake of ascorbic acid ranged from 10.4 mg. to 13.8 mg. per day. The whole experimental period was divided into :—

(1) *Saturation period.*—Five days. For four days 200 mg. of ascorbic acid (Redoxon)* was given to each subject. On the fifth day a test dose of 400 mg. was administered orally and if the 24-hour urinary excretion of ascorbic acid showed the value as 50 per cent or more of the total intake for that day, the subject was considered to be saturated.

(2) *Experimental level period.*—After the subjects had been saturated, they were given 100 mg. dose of ascorbic acid daily along with their diets for six days. On the seventh day, 400 mg. test dose of ascorbic acid was again administered and if 50 per cent of this test dose excreted in the urine, the subjects were considered to be saturated at the level of 100 mg. intake for the previous six days. If the subjects showed evidence of saturation at this level of 100 mg. intake, they were given the lower doses of 75 mg. and 85 mg. in two more experimental periods. By repetition of this process, the saturation level of ascorbic-acid requirement for an individual subject was determined.

Urine was generally collected during the 24-hour period preceding and following the test dose after saturation and experimental periods. In order to observe the gradual change in the urinary output of ascorbic acid after different doses, daily collection was also made up to the two saturation periods and first experimental period.

The ascorbic acid of foods and urine was estimated by the indophenol-titration method using metaphosphoric acid.

RESULTS AND DISCUSSION.

The summary of results are shown in the Table and it is observed from the data presented in it that none of the subjects became saturated at the first 5-day period of saturation. They were, therefore, continued to the second 5-day period of saturation after which they showed evidences of saturation.

* Acknowledgment is made to Messrs. Volkart Brothers, Roché Scientific Division, Bombay, for a generous supply of ascorbic-acid Redoxon tablets.

TABLE.

The figures indicate the 24-hour urinary response in test dose of 400 mg. of ascorbic acid (total intake = 400 mg. + amount in diet) following saturation period and experimental level periods of 75-mg., 85-mg. and 100-mg. doses.

Subject.	Dietary intake of ascorbic acid.	SATURATION LEVEL PERIODS.				EXPERIMENTAL LEVEL PERIODS.			
		PERIOD I. 200-mg. dose.		PERIOD II. 200-mg. dose.		PERIOD I. 100-mg. dose.		PERIOD II. 75-mg. dose.	
		Total excretion in mg. on test dose of 400 mg.	Excretion as per centage of total intake.	Total excretion in mg. on test dose of 400 mg.	Excretion as per centage of total intake.	Total excretion in mg. on test dose of 400 mg.	Excretion as per centage of total intake.	Total excretion in mg. on test dose of 400 mg.	Excretion as per centage of total intake.
B.S. Body-weight—49 kilograms.	10.4	142.5	34.7	265.0	64.6	220.3	53.7	160.0	39.0
M.R. Body-weight—48 kilograms.	13.1	110.6	26.7	273.5	66.2	331.3	80.2	241.5	58.4
A.D. Body-weight—45 kilograms.	13.8	88.1	21.3	305.1	73.7	273.1	66.0	330.0	79.7
B.M. Body-weight—53 kilograms.	12.6	120.0	20.1	247.0	59.9	286.6	69.5	125.0	30.3
S.T. Body-weight—46 kilograms.	11.9	116.3	28.7	272.4	66.2	279.9	68.0	150.0	36.4

After saturation when the experimental level dose of 100 mg. was given to the subjects, all the subjects were found to be saturated at this level of intake. After this they were kept on 75-mg. level of intake and at this level only one subject, M. R., became saturated. They were continued to the third experimental level period with doses of 85-mg. ascorbic acid per day and in this case it was found that two subjects M.R. and A.D. became saturated.

Thus, it is observed that the saturation requirement of subjects B.S., B.M. and S.T. is between 85 mg. and 100 mg. ; that of A.D. between 75 mg. and 85 mg. and that of M.R. in the range of 75 mg.

This wide individual variation in the ascorbic-acid requirement for saturation is minimized to a great extent when calculated on the basis of per kilogram body-weight. On compilation in this way the saturation requirement comes within the limit of 1.7 mg. to 2.0 mg. in cases of the subjects B.S. B.M. and S.T., 1.7 mg. to 1.9 mg. in case of the subject A.D. and 1.6 mg. in case of the subject M.R.

SUMMARY.

By using the modified method of urinary response to test dose, the saturation requirement of ascorbic acid of five Indian adults have been studied.

Three subjects required 85 mg. to 100 mg., one subject required 75 mg. to 85 mg. and another required in the range of 75 mg. of ascorbic acid for saturation.

The daily requirements of vitamin C for saturation in the five subjects investigated varied between 1.6 mg. and 2.0 mg. per kilogram of body-weight.

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NUTRITIVE VALUE OF SOME OF THE WEST COAST MARINE FOOD FISHES OF THE MADRAS PROVINCE.*

BY .

S. T. CHARI.

(From the Fisheries Technological Station, Calicut.)

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THE investigations on the nutritive values of the various food fishes, both marine and inland, have recently been undertaken by Indian workers. Saha and Guha (1939) and Basu and De (1938, 1938a) have investigated the food values of fresh-water fishes of Bengal, while Niyogi *et al.* (1941) and Setna *et al.* (1944) have worked on the marine fishes of the Bombay Province and the latter have conducted the investigation on almost all fishes representative of the catches of the coast. It seemed desirable to carry out a similar study on the marine food fishes of the west coast of the Madras Province and the details given below deal with the nutritive values of about 34 important marine food fishes including shell fishes.

METHODS.

The chemical or proximate analysis is mainly on the edible portions of the fishes. In small fishes, however, as silver-bellies, soles and white bait (which are usually eaten whole), the analysis was conducted on the whole fish including the bones and heads after removing only the viscera which formed a very negligible portion. In others, the head, fins, scales, skin, skeletal structures and viscera were discarded and the flesh collected together and analysed. The analysis was done only on absolutely fresh fishes, almost immediately after landing.

Sampling.—The method of sampling was the same as described by the Association of Official Agricultural Chemists (1945). For medium-sized fishes, one longitudinal half from each of the 5 to 10 fishes under study was taken and minced thrice in a food chopper and mixed well. In the case of a large fish, three transverse slices, 1" thickness, were cut from each of the fishes, one slice from the back immediately after the pectoral fin, the second slice half-way between the first

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TABLE
Analysis of

Number.	Local name (Malayalam).	Common English name.	Scientific name.	Weight of an average fish analysed.
1	Sravu ...	Shark	<i>Carcharias laticaudus</i>	2 lb.
2	Sravu ...	Shark	<i>Carcharias menisorrh</i>	2 "
3	Neithirandi ...	Ray	<i>Rhinoptera seveli</i>	4 "
4	Aila ...	Mackerel	<i>Rastrelliger kanagurta</i>	3.5 oz.
5	Vala manthal ...	Sole	<i>Cynoglossus semifasciatus</i>	0.5 "
6	Nalla mullan ...	Silver-belly	<i>Leiognathus bindus</i>	0.25 "
7	Chakra mullan ...	Silver-belly	<i>Leiognathus insidiatrix</i>	0.25 "
8	Valial etta ...	Cat fish	<i>Arius dussumieri</i>	7 lb.
9	Palli kora ...	Jew fish	<i>Otolithes ruber</i>	4 oz.
10	Kora ...	Jew fish	<i>Pseudosciaena coiber</i>	$\frac{1}{2}$ b.
11	Aya kora ...	Scor	<i>Cybinus guttatum</i>	12 "
12	Velli thalayan ...	Ribbon fish	<i>Trichiurus suvala</i>	2 oz.
13	Nalla mathi ...	Oil sardine	<i>Sardinella longiceps</i>	2 $\frac{1}{2}$ "
14	Chala mathi ...	Sardine	<i>Sardinella fimbriata</i>	1 $\frac{1}{2}$ "
15	Ambatta ...	Sardine	<i>Opisthopterus tartoor</i>	$\frac{1}{2}$ "
16	Nethal ...	White bait	<i>Stolephorus tri</i>	1/14 "
17	Chamban ...	Goggler	<i>Caranx crumenophthalmus</i>	3 "
18	Manangu ...	Anchovy	<i>Engraulis myslox</i>	2 "
19	Ovupara ...	Horse mackerel	<i>Caranx malampygus</i>	4 "
20	Adavu ...	Big-jawed jumper	<i>Lactarius lactarius</i>	1 $\frac{1}{4}$ "
21	Karuppu avoli ...	Black pomfret	<i>Stromateus niger</i>	1 lb.
22	Vellai avoli ...	White pomfret	<i>Stromateus cinereus</i>	1 $\frac{1}{2}$ "
23	Sooda ...	Tunny	<i>Thynnus macropterus</i>	3 "
24	Bahmeen ...	Thread fin	<i>Polynemus heptadactylus</i>	1 oz.
25	Thirutha ...	Mullet	<i>Mugil ocar</i>	3 lb.
26	Narimeen ...	Cockup	<i>Lates calcarifer</i>	2 "
27	Valathan ...	Ox-eyed herring	<i>Megalops cyprinoides</i>	1 "
28	Kannan mathi ...	Indian herring	<i>Pellona brachysoma</i>	3 oz.
29	Poozhan ...	Indian whiting	<i>Sillago sihima</i>	3 "

I.

fresh fish.

Average length in cm.	Edible portion, per cent.	H ₂ O, per cent.	Protein, per cent.	Fat, per cent.	Ash, per cent.	P ₂ O ₅ , per cent.	CaO, per cent.	Iron, mg. per 100 g.
40	62.5	75.54	22.93	0.69	1.44	0.58	0.52	1.43
43	66.6	75.95	21.59	0.42	1.24	0.42	0.23	1.25
30	50.0	75.25	20.91	0.50	1.24	0.63	0.25	5.30
21	61.6	77.30	18.92	1.69	1.58	0.69	0.61	4.45
12	100.0	72.38	19.48	4.70	3.14	1.19	1.54	10.14
8	95.0	76.02	18.77	1.16	3.57	1.52	1.51	1.73
7	94.0	76.38	19.17	1.55	3.20	1.73	1.04	2.20
62	58.0	79.68	17.36	0.60	1.06	0.46	0.38	9.16
21	49.0	77.01	19.95	2.22	1.62	0.61	0.30	4.82
27	75.0	78.32	18.76	0.81	1.91	0.67	0.43	4.44
141	66.0	72.71	22.45	4.00	1.49	0.80	0.12	5.37
46	82.0	76.61	18.11	3.24	1.51	0.47	0.30	13.87
18	70.0	76.49	19.57	2.03	1.79	0.79	0.47	6.09
15	60.0	77.23	20.84	1.93	1.67	0.64	0.29	9.19
11	49.0	78.30	18.16	0.15	2.05	0.52	1.49	4.92
6	100.0	79.11	14.52	1.38	2.46	0.96	0.92	3.87
14	60.0	76.88	18.66	1.61	1.76	0.78	0.60	8.21
16	66.0	69.26	19.27	9.64	1.56	0.43	0.17	1.52
23	58.0	76.87	21.20	1.55	1.28	0.58	0.50	2.01
16	66.6	73.33	19.39	5.78	1.42	0.73	0.33	3.35
39	70.0	74.50	20.30	2.60	1.14	0.70	0.44	2.29
30½	68.0	78.39	17.02	1.30	1.54	0.58	0.25	12.23
36	61.0	71.94	23.84	1.59	1.80	0.82	0.61	6.78
15	62.5	76.59	17.94	2.86	1.34	0.56	0.44	7.29
59	73.5	69.92	19.06	7.83	1.09	0.44	0.52	4.38
33	50.0	78.16	18.37	0.95	1.74	0.72	0.56	4.13
39	73.2	74.71	20.65	2.16	1.58	0.26	0.56	6.30
16	50.0	72.82	20.28	3.20	1.45	0.67	0.57	9.31
35	61.3	77.14	19.17	0.60	1.53	0.58	0.10	2.20

slice and the vent, and the third, immediately after the vent. The bones were separated and the muscular tissue minced and mixed as above. In the case of prawns, the head and the outer shells and the viscera were removed and the pulp was analysed. The shells of the blue mussels (a bi-valve) were opened by inserting a sharp knife, the meat along with its juice scooped into a beaker, minced and analysed.

Estimations.—The moisture content was determined by drying a weighed sample of the minced pulp to a constant weight in a steam oven at 100°C., the crude protein by multiplying the percentage of nitrogen as determined by the Kjeldahl method, by the factor 6.25, ash by the Stolte's dry-ashing method as applied by Tisdall and Kramer (1921), and fat by extracting the dried sample with pure anhydrous ethyl ether for about 12 to 16 hours in a Soxhlet apparatus. The ash extracted with hydrochloric acid was further analysed for phosphorus, calcium, and iron contents: P by the ammonium-molybdate volumetric method (Burns and Henderson, 1935), calcium by the McCrudden's (1911-12) permanganate method, and iron by the Elvehjem (1930)-Kennedy (1927) method.

Table I gives the vernacular (Malayalam), the popular English and the scientific names of the fishes as well as the percentages of edibility and of the other chemical constituents.

The nutritive values of almost all the fishes under study, as shown in Table I, are high showing about 15 to 22 per cent of protein on a moisture basis of about 75 per cent, a fair quantity of mineral matter as phosphorus, calcium and iron, and varying quantities of fat. The carbohydrate content of fishes is ordinarily negligible. The average calorific value of a fish (by using the factor 9 for fat and 4 for proteins and carbohydrate) is about 120 calories per 100 g. The vitamin content of fish has not been determined in this Laboratory.

An examination of Table I will show that the tunny fish stands first in its high protein content followed by sharks and rays, and then by other *clupeids*. Anchovies stand first in the fat contents closely followed by the mullet and big-jawed jumper. Other fatty fishes worthy of mention are oil sardine, seer, sole, ribbon fish and the Indian herring. The ash contents of the fishes analysed whole (like silver-belly, sole and white bait) stand high naturally due to the skeletal structures which are made up mainly of phosphorus and calcium as shown by the sub-analysis of the ash for the above minerals. The other fishes vary in their P_2O_5 and CaO contents from 0.5 to 0.9 per cent and 0.2 to 0.7 per cent, respectively. The iron content in almost all the cases is less than 10 mg. per cent with a few exceptions which go up to about 13 mg. per cent. On the whole the various analyses by the method described above compare favourably with the analyses done by other workers in the field.

Table II contains the analyses of four species of prawns and a bi-valve, viz. blue mussel.

On a review of Table II, among the prawns, the species *Peneopsis dobsonii* attains a fairly big size and the analytical values also show its superiority over others of the same class. The blue mussel (*Mytilus edulis*) gives about 10 per cent of protein on a moisture basis of 81.6 per cent which works up to about 55 per cent of protein compared to about 70 per cent in the case of fishes, on a moisture-free

TABLE II.

Analyses of shell fishes.

Number.	Local name (Malayalam).	Common English name.	Scientific name.	Average number per lb.	Edible portion, per cent.	Average length in cm.	H ₂ O, per cent.	Proteins, per cent.	Fat, per cent.	Ash, per cent.	P ₂ O ₅ , per cent.	CaO, per cent.	Iron, mg. per 100 g.
1	Chemmin	...	<i>Peneus monodon</i>	80	43.0	11	78.46	17.64	0.41	1.54	0.79	0.40	4.66
2	Kozhi chemmin	...	<i>Pencopsis dohsonii</i>	3	46.6	31	78.87	18.01	0.42	1.68	0.77	0.22	9.42
3	Chemmin	...	<i>Peneus semisulcatus</i>	5	52.3	19	76.70	20.76	0.69	1.49	0.75	0.39	6.92
4	Vale chemmin	...	<i>Trachypeneus asper</i>	40	50.0	11	78.59	18.74	0.85	1.23	0.60	0.26	1.80
5	Kaduka	...	<i>Mytilus edulis</i>	35	42.8	5	81.46	9.92	1.97	3.04	0.36	2.59	8.02

basis. The ash of the bi-valve flesh is also high due to the high calcium content. Besides, the shell fishes contain a fair amount of carbohydrate unlike ordinary fishes. No analysis of their vitamin contents has been done.

A survey of the two tables (Tables I and II) will show that there are wide variations in almost all the values for the various species of fish analysed. It is also known that the chemical composition of the same species of fish may vary with reference to season, reproductive cycle, etc., and a study on this seasonal variation in the case of one particular fish has already been taken up. The analytical tables may be useful for comparison with other foods when planning a satisfactory balanced diet. The tables will also show that in their nutritive values these fishes compare favourably with those from other parts of India and elsewhere.

The culinary and gustatory properties of the fish are among the main factors which add weight to the contention that it can be a potential source of universal nutritive food. Being devoid of any tough ligaments and tissues, it is easily cooked, chewed and digested.

A word about the food values of the few ordinary species of despised fishes like sharks, rays, etc., will not be out of place. These fishes contain a greater percentage of protein than most other fishes and possess very favourable qualities like colour, flakiness and consistency besides their low cost. At present these fishes are not very popular in some localities due to some religious and sentimental objections, whereas in other countries these are in much demand. It is high time that such prejudices are cast off and more universal use is made of these cheap sources of fish food.

SUMMARY.

Thirty-four species of fishes including shell fishes have been analysed for water, protein, fat, ash, P_2O_5 , CaO and iron. The local name, scientific name, average weight, size, and percentage of edible portions are also given.

There are wide variations in the chemical composition of the various fishes under study. Protein content varies from 15 to 23 per cent, tunny having the highest protein content of 23.84 per cent. Fat varies from 0.5 to 9.64 per cent with the anchovies at the top of the list. Ash varies from 0.4 to 3.57 per cent, maximum being found in the silver-belly which was analysed with the skeletal structures, and the lowest being found in the prawns.

The fishes eaten whole (like silver-belly, sole and white bait) and the shell fish (*Mytilus edulis*) show a high phosphorus and calcium content in their ash.

The iron content of almost all fishes was found to be below 10 mg. per cent excepting a few cases which touched to about 13 mg. per cent.

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INVESTIGATIONS ON BIOLOGICAL VALUE OF CEREAL MIXTURES IN A RICE EATER'S DIET BY HUMAN FEEDING TRIALS.

BY

K. MITRA,

S. K. VERMA,

AND

S. AHMED.

(From the Nutrition Scheme, Public Health Laboratories, Patna.)

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INTRODUCTION.

WITHIN recent years the shortage of food grains had necessitated the introduction of locally non-familiar cereals in certain parts of India. Wheat, a very popular article of diet in North-Western India, was comparatively unknown as staple food in South India. The millets, Bajra (*Pennisetum typhoideum*), Junera (*Sorghum vulgare*) and Marooa (*Eleusine coracana*), were unknown in Bengal. Wheat had to be introduced in Madras and bajra in Bengal due to extreme shortage of rice in both the provinces. Again, owing to shortage of supplies, the so-called inferior cereals (barley, maize or millets) had to be mixed from time to time in small proportions with wheat flour.

From a study of the table of food values it was very justifiably advocated by the nutrition workers in India that a mixture of cereals in the diet would improve its nutritive value as compared to a diet consisting of rice or any other cereal alone. Interference with conventional dietary habits of humans has often been resented in varying degrees and this happened in India also. With a view to answer some of the questions raised with introduction of unfamiliar cereals the present investigation was undertaken. The points at issue involved in some of the eastern and southern provinces may be summarized under two heads: (a) what was the biological value of proteins in an average rice eater's diet, and (b) how far this value was affected when part of the rice quota was replaced by whole-wheat flour or wheat

flour mixed either with maize or millets or barley. Feeding trials were conducted on six healthy adult human (male) subjects who were carrying out their normal duties during the period of investigation. The cereal quota in the usual home diets of these subjects, belonging to the province of Bihar, consisted mostly of rice and small quantities of wheat.

EXPERIMENTAL.

Six adult human subjects, M. R., B. M., S. A., I. L., S. L. and P. M., were kept on each of the seven test diets for a period of 8 days. Each of them were in good health and continued in their duties throughout the experimental period. The test diets very nearly resembled the usual food consumed by each of the subjects in their own homes. An average test diet consisted mainly of cereals (550 g. to 800 g.), pulse or dhal (75 g.) as gruel, potatoes and small servings of vegetables. No milk or flesh foods were consumed. One of the subjects (S. A.) was habituated to tea drinking but he voluntarily abstained from tea habit more for maintaining uniformity than anything else.

For the estimation of endogenous nitrogen metabolism the subjects were kept on a 'low nitrogen diet' instead of a 'nitrogen-free diet'. The estimation was done in the case of three subjects only (B. M., S. L. and P. M.), the data for others were available as they had been kept on low nitrogen diet a few months previously in connection with another feeding trial. The reasons for preferring the low nitrogen diet and the details of technique have been described in a previous communication from this Laboratory (Mitra and Verma, 1947).

TABLE I.

Data for low nitrogen diet.

Name of subjects.	Age in years.	Weight of the subject, kg.	Total dry weight of food, g.	Weight of nitrogen in food, g.	. OUTPUT OF NITROGEN.		
					Urine, g.	Faeces, g.	Total, g.
M. R. ...	22	41.0	820	0.31	1.414	0.710	2.124
B. M. ...	22	41.2	825	0.33	1.408	0.723	2.131
S. A. ...	40	52.5	990	0.50	1.899	1.220	3.119
I. L. ...	31	48.0	990	0.50	1.487	1.170	2.657
S. L. ...	25	44.6	860	0.36	1.461	1.042	2.503
P. M. ...	22	42.5	860	0.36	1.464	0.916	2.380

After the low nitrogen period the subjects were put on the different test diets.

During the first 8-day period the test diet consisted of boiled rice (the cooking water being thrown away), dhal or the cooked preparation of the most popular pulse red gram (*Cajanus indica*). The sample of rice used did vary from time to time but it was always of the same type: medium quality parboiled hand-pounded. For all purposes it may be assumed that the protein value or the table qualities did not materially differ. The pulse constituted about 13 to 14 per cent by weight of the total rice consumed. This was the proportion the subjects were accustomed to and liked best. Small servings of mashed potatoes, and vegetables consisting mostly of bottle-gourd (*Lagenaria vulgaris*) or vegetable marrow (*Luffa ærægyptica*) and edible green leaves, were also permitted. The cooking of vegetables was done with mustard oil and very small amounts of condiments. The protein from vegetables and curry rarely exceeded 10 per cent of the total daily intake. The pulse and vegetable quota was kept constant in the subsequent test diets. After the first 8-day period, part of the rice in the diet was replaced with wheat flour made into chapatti or unleavened bread. The chapattis were smeared with ghee (butter fat) to make them palatable and served hot with the evening meals. Twenty-four-hour collections of urine and stool were made and the nitrogen estimated on the lines reported in the previous communication (Mitra and Verma, *loc. cit.*). Estimation of nitrogen in the aliquot portions of test diets was also made. The composition of the test diet during the 1st and 2nd weeks is detailed in Table II:—

TABLE II.

Composition of test diets.

Name of subjects.		1ST TEST PERIOD (RICE ALONE).		2ND TEST PERIOD (RICE AND WHEAT MIXED).		
		Rice, g.	Pulse, g.	Rice, g.	Wheat flour, g.	Pulse, g.
M. R.	550	75	275	234	75
B. M.	550	75	275	234	75
S. A.	800	110	400	300	110
I. L.	800	110	400	300	110
S. L.	650	90	325	255	90
P. M.	650	90	325	255	90

In addition to the above, the test diet included cooking fat and butter fat (ghee) 35 g. to 45 g., potatoes 100 g. to 120 g., bottle-gourd or vegetable marrow 140 g. to 150 g., and leafy vegetables 125 g. to 150 g. No vitamin supplements were given.

During the subsequent test periods the only alteration in the diet being the replacement of 25 per cent by weight of the wheat flour quota by an equivalent weight of flours from barley, maize, junera, marooa and bajra in succession. Special allocations of the different grains were received in bulk periodically through the courtesy of the local controller of rationing. The grinding of flour from wheat, barley, maize and millets and the proper blending thereof in the proportion 3:1 with other cereals was carried out under the personal supervision of one of the authors (S. K. V.) and the subjects were never told about the constitution of the mixed flours used for the preparation of chapatti. At times two of the subjects did mention that the taste and flavour of the chapatti was not similar to that supplied during the previous week. They were told that the supply of flour happened to be purchased from ration shops in the different weeks, hence the difference. The subjects were aware that the licensed ration shops had no control over the source of supply and slight difference in colour, texture or flavour of flour was a usual occurrence. Within reasonable limits attempt was made to maintain a uniform level of protein intake in the different test periods. The make up of cereal and pulse quota of the diets in the 3rd, 4th, 5th, 6th and 7th test periods is given in Table III :—

TABLE III.

Main ingredients of diets from 3rd to 7th test periods.

Name of subjects.	Rice, g.	Wheat flour, g.	Supplementary flour used for blending, g.	Pulse, g.
M. R. ...	275	180	60	75
B. M. ...	275	180	60	75
S. A. ...	400	240	80	110
I. L. ...	400	240	80	110
S. L. ...	325	204	68	90
P. M. ...	325	204	68	90

Column 4 of Table III indicates the weight in grammes of the supplementary (cereal) flours used for blending with wheat flour. During each of the test periods from 3rd to the 7th, the different mixed flours used for the preparation of chapatti consisted of wheat flour and one other supplementary cereal flour. The supplementary cereal used was barley in the 3rd, maize in the 4th, junera in the 5th, marroa in the 6th and bajra in the 7th test periods.

RESULTS AND DISCUSSION.

Owing to shortage of accommodation in the hut adjoining the laboratory, which the subjects made their temporary abode during the experimental periods, only two subjects could be taken up at any one time. Consequently, the experiments were stretched over a period of more than a year. The samples rice, wheat, barley, maize and millets used were not always the same but as close an approximation as could be possible, with each type of cereal samples used in the test diets, was maintained. The nitrogen intake and excretion data of the subjects with the seven different test diets are given in Table IV :—

TABLE IV.

Intake of nitrogen and excretion on test diets.

Name of subjects.	Weight of the subject, kg.	Weight of nitrogen in food, g.	OUTPUT OF NITROGEN.				
			Urine, g.	Fæces, g.	Total, g.	Balance, g.	
<i>Diet containing rice only.</i>							
M. R. 	41.0	11.32	4.371	2.894	7.265	+4.005	
B. M. 	41.2	11.32	4.623	3.001	7.624	+3.696	
S. A. 	52.5	13.76	5.765	3.801	9.565	+4.105	
I. L. 	48.0	13.76	4.987	3.778	8.765	+4.995	
S. L. 	44.6	11.64	4.835	2.788	7.623	+4.017	
P. M. 	42.5	11.64	4.625	3.035	7.660	+3.980	

J, MR

TABLE IV—*contd.*

Name of subjects.	Weight of the subject, kg.	Weight of nitrogen in food, g.	OUTPUT OF NITROGEN.			
			Urine, g.	Fæces, g.	Total, g.	Balance, g.

Diet containing rice and wheat only.

M. R.	12.86	5.830	3.263	9.092	+3.767
B. M.	12.86	5.809	3.548	9.357	+3.503
S. A.	15.81	7.954	4.536	12.490	+3.320
I. L.	15.81	6.867	4.105	10.972	+4.838
S. L.	13.59	6.448	3.679	10.125	+3.465
P. M.	13.59	6.272	3.892	10.164	+3.426

Diet containing rice, wheat and barley only.

M. R.	12.48	5.407	3.331	8.738	+3.742
B. M.	12.48	5.117	3.896	9.013	+3.467
S. A.	15.46	7.124	4.633	11.757	+3.703
I. L.	15.46	6.076	4.547	10.623	+4.837
S. L.	13.46	5.753	3.411	9.164	+4.296
P. M.	13.46	5.884	3.548	9.432	+4.028

Diet containing rice, wheat and maize only.

M. R.	12.60	5.453	3.712	9.165	+3.435
B. M.	12.60	5.815	3.836	9.651	+2.949
S. A.	15.52	7.510	3.279	10.789	+4.734
I. L.	15.52	6.751	4.293	11.044	+4.476
S. L.	13.68	6.118	3.942	10.060	+3.620
P. M.	13.68	5.938	4.144	10.082	+3.598

TABLE IV—concl'd.

Name of subjects.	Weight of the subject, kg.	Weight of nitrogen in food, g.	OUTPUT OF NITROGEN.			
			Urino, g.	Fæces, g.	Total, g.	Balance, g.

Diet containing rice, wheat and junera only.

M. R.	10.15	5.581	2.119	7.700	+2.450
B. M.	10.15	5.327	1.909	7.226	+2.914
S. A.	15.38	7.916	3.744	11.660	+3.720
I. L.	15.38	7.198	4.014	11.212	+4.168
S. L.	13.60	6.124	4.116	10.240	+3.360
P. M.	13.60	6.497	3.786	10.273	+3.327

Diet containing rice, wheat and marooa only.

M. R.	12.38	5.316	2.359	7.875	+4.505
B. M.	12.38	5.176	2.463	7.639	+4.741
S. A.	15.86	7.566	3.916	11.482	+4.378
I. L.	15.86	7.076	4.221	11.297	+4.563
S. L.	13.54	5.813	3.537	9.350	+4.190
P. M.	13.54	5.993	3.570	9.563	+3.977

Diet containing rice, wheat and bajra only.

M. R.	12.32	5.737	3.006	8.743	+3.577
B. M.	12.32	5.260	2.583	7.843	+4.477
S. A.	15.67	8.043	3.877	11.920	+3.750
I. L.	15.67	7.032	4.468	11.500	+4.170
S. L.	13.66	6.180	3.729	9.909	+3.751
P. M.	13.66	6.010	3.211	9.221	+4.439

The relative biological value of the proteins in the different diets have been calculated according to the conventional formula described fully by Chick *et al.* (1935). The mean biological value was found to be the highest (66.6 per cent) when rice constituted the sole source of cereal in the diet and lowest (54.5 per cent) when rice with a mixture of wheat and junera flour was consumed. The figures for a diet consisting of rice and pure wheat flour were found to be slightly higher than that of wheat junera mixture. In the last column of Table V one of the subjects (B. M.) has recorded a rather high figure as compared to others. On inquiry it could be ascertained that this particular subject of all others was accustomed in his home to bajra in chapatti or in any other form for appreciable periods. There is a common belief that familiarity of the gastro-intestinal tract with any particular type of food is likely to exert a favourable action on the process of digestion and assimilation. It is not claimed that this one finding supports the belief mentioned above. It was, however, felt by the authors that this fact deserved a mention for whatever it was worth.

Basu *et al.* (1941) have in their human feeding trials on a vegetarian rice diet recorded a biological value of 67 per cent (one subject only) and 58 and 63 per cent (in two subjects) on a whole-wheat diet. In the present series of experiments the figures for rice diet (66.6 per cent) closely follow theirs (67 per cent) but in the case of wheat diet their figures were slightly higher in one case and almost of the same order in the second subject.

TABLE V.
Biological values per cent of mixed proteins.

Name of subjects.	Rice only.	Rice and wheat.	Rice, wheat and barley.	Rice, wheat and maize.	Rice, wheat and junera.	Rice, wheat and marooa.	Rice, wheat and bajra.
M. R. ...	67.7	56.2	59.5	57.9	52.3	63.6	56.9
B. M. ...	64.5	56.3	60.2	53.5	56.2	63.7	63.2
S. A. ...	65.5	51.6	56.7	57.6	53.3	57.0	52.7
I. L. ...	68.6	56.2	62.0	57.6	54.4	57.1	55.2
S. L. ...	65.9	55.4	61.3	56.8	55.7	61.5	56.6
P. M. ...	66.8	54.7	58.8	57.2	53.1	58.4	60.0
MEAN ...	66.6	55.1	59.8	56.8	54.1	60.2	57.4

The present investigation lends support to the belief prevalent amongst nutrition workers in India that a mixture of maize and millets in the wheat flour tends to increase its biological value in the cereal predominating vegetarian diet consumed by millions in India. Judging from the biological value of the protein alone it appears that rice protein has the highest value. For a 25 per cent mixture with wheat flour (in the preparation of chapatti) the cereals in order of preference seem to be marooa, barley, bajra, maize and finally junera. Though in the case

of junera it is doubtful whether the wheat flour improves at all in the scale when junera is mixed. The respective figures for digestibility coefficient have also been worked out for the different test diets and are shown in Table VI:—

TABLE VI.

Figures for the digestibility coefficients of mixed proteins.

	M. R.	B. M.	S. A.	I. L.	S. L.	P. M.	Average.
Rice alone ...	80	80	80	81	85	82	81
Rice and wheat ...	80	78	79	80	81	78	79
Rice, wheat and barley ...	79	75	78	78	82	80	79
Rice, wheat and maize ...	76	75	86	80	79	76	79
Rice, wheat and junera ...	85	88	80	80	77	79	82
Rice, wheat and marooa ...	87	86	83	80	82	80	83
Rice, wheat and bajra ...	80	85	83	80	78	83	82

The digestibility coefficients do not differ appreciably from one another and are almost of the same order as recorded by Basu *et al.* (*loc. cit.*) with mixed proteins in rice and wheat diets.

SUMMARY.

Relative biological value of mixed proteins in the poor quality Indian diet lacking in milk and flesh foods have been investigated by human feeding trials. The diets consisted of rice, wheat, pulse and vegetables. Part of the wheat in some of the test diets was replaced by other cereals and, except in one case, such a mixture in the wheat flour has tended to increase its biological value.

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STUDIES ON ANTIRABIC VACCINES.

Part I.

IMMUNIZING VALUE OF ANTIRABIC VACCINE.

BY

R. SANJIV RAO, M.B.,

AND

J. R. DOGRA, M.D.

(*From the Haffkine Institute, Bombay.*)

[Received for publication, May 15, 1948.]

THE utility of antirabic vaccines as prophylactic agents has remained in doubt despite their use for such a long time in Pasteur Institutes the world over. For long, the main evidence was based on statistical analysis of cases of individuals treated with different antirabic vaccines. The low mortality rate amongst those treated with vaccine was taken as an index of the efficiency of the vaccine. McKendrick (1940) in his masterly and laborious analysis brought out all available statistical evidence on the subject from data collected from Pasteur Institutes all over the world. Greenwood (1946) who completed this statistical survey commented upon it from the point of view of its utility for assessing the value of the rabies prophylaxis. He (p. 363) summed up thus: 'It would be idle to pretend that the results are satisfactory to me or likely to be satisfactory to readers. The expectation of those who planned the investigation twenty years ago has not been fulfilled. I do not believe further massing of data will lead to fulfilment.'

Long before Greenwood's conclusions, workers in India were making attempts to determine the extent and duration of immunity conferred by rabies prophylaxis in animals. Cunningham and Malone (1930) vaccinated rabbits after corneal infection with street virus. Of the 44 rabbits inoculated but subsequently not treated all died of rabies. Of 180 similarly infected but subsequently treated with etherized or phenolized vaccine all died of rabies but one. In further experiments (Cunningham, Malone and Craighead, 1933) on monkeys, treatment resulted in percentage mortalities of 58.2 per cent of the total 432 animals as compared with

72.2 per cent in the control batches of untreated monkeys. These mortality figures while coming within the limits of statistical significance did not show an appreciable difference especially in view of the fact that the authors recorded 5 to 20 per cent deaths due to extraneous causes.

Shortt, Malone, Craighead and McGuire (1934) using the neck-muscle-injection technique for inducing rabies showed that in five experiments of 197 inoculated animals 18.8 per cent succumbed to rabies as compared with 70.6 per cent of untreated monkeys. From these experiments it was inferred that antirabic vaccine had a very definite immunizing value when immunization preceded infection.

Covell, McGuire, Stephens and Lahiri (1936) published data of 24 tests. In the first experiment of 92 monkeys contained in the 9 batches, 48 (52 per cent) succumbed following treatment as compared with 10 out of 10 (100 per cent) controls. Two further experiments showed a mortality rate of 22 and 20 per cent amongst the inoculated and 77 and 76 per cent amongst the uninoculated monkeys. They, however, emphasized the difficulties encountered in antirabic work and warned against hasty conclusions from isolated experiments.

Webster (1939*a*) reviewed the existing literature on the subject and concluded that all workers, except Fermi whose work had not been confirmed, had failed to demonstrate a significant protective effect against vaccination following experimental exposure to rabies virus by any route in animals. Also that vaccine, virulent or non-virulent, given before exposure, had generally been found effective against test virus provided that (*a*) the test virus was given peripherally rather than centrally and in amounts fatal to less than 100 per cent of control, and (*b*) the vaccine employed was given in multiple doses and in amounts not less than 1 per cent of the body-weight. The results of experiments were, however, considered by him to be irregular, showing meagre immunizing power of the vaccines and little superiority of one preparation over the other. This situation led Webster (1939*b*) to develop a quantitative and practical mouse-potency test. He found that Swiss-W strain of mice were 'highly susceptible', 'relatively uniform in their response to rabies virus', that they contracted the 'classical type of disease' after injection of a very small amount of virus and that they were 'readily immunizable' against measurable doses of virus given intracerebrally or intramuscularly. Monkeys and dogs used by previous workers were no longer required. They were anyhow expensive, difficult to obtain always in sufficient numbers, difficult to handle and look after, besides being quite unsuitable for routine tests for standardization of rabies vaccines. The new Webster's mouse test opened wide fields for further work on the subject.

Habel (1940) further substantiated and modified the mouse-test technique and laid down a standard method. Using his technique several determinations have been made since 1946 in the Department of Virus Diseases of this Institute. Various aspects of the rabies problems have been under investigation.

The present communication submits our results of mouse tests on 5 per cent Semple's vaccine which is prepared at this Institute. One brew of chloroformed and phenolized vaccine prepared from rabbit brain for purposes of comparison was tested. Samples of antirabic vaccines from various Pasteur Institutes in India were obtained and assayed.

MATERIALS AND METHOD.

The vaccine under test was 5 per cent Semple's phenolized vaccine of dead virus (Paris strain) prepared from sheep's brain with a final carbolie content of 0.5 per cent. Large quantities of this vaccine are prepared at this Institute. Random samples were taken for test extending over 1946, 1947 and 1948.

Haffkine Institute inbred strain of white mice, four weeks old, each weighing 16 g. to 18 g. was used.

Paris strain of rabies virus has been in use in this Laboratory since 1932. For test-virus infection this stock strain was once passaged through three one-month old white mice. The mice were killed immediately on the first day of the appearance of signs of paralysis, usually the 7th day after infection, their brains dissected and stored in a refrigerator at 0°C., and used within 2 to 3 days for test-virus challenge infection.

The vaccine batches under test were diluted 1 in 10 in normal saline. Of this 0.25 c.c. was injected intraperitoneally into mice on alternate days for 6 days. The control group of mice was set alongside. Fourteen days after the administration of the first dose of vaccine, the vaccinated mice and controls were given the test-virus challenge infective dose intracerebrally. For this purpose the rabid mouse brains stored in refrigerator were emulsified and a 10-per cent suspension made in horse serum. The horse serum was diluted 1 in 10 in distilled water. The mouse-brain suspension was then centrifuged at 1,000 r.p.m. for 10 minutes, the supernatant was pipetted off with a sterile pipette and serial dilutions made in 10-per cent horse serum. The mice already immunized and divided into groups were then given this test-virus challenge infective dose, 0.03 c.c. of each dilution, the animals being etherized to facilitate intracerebral injection. It may be noted that with the special $\frac{1}{4}$ -inch long needles (27 gauge) and by applying gentle pressure over the site of inoculation which was in the middle line midway between the ears and eyes of the mouse, leakages were prevented and successful inoculations achieved.

The animals were then observed for signs of paralysis for 20 days. Animals that died from extraneous causes were excluded and only those showing signs of paralysis considered having succumbed to rabies infection. At the end of 20 days total survivors in each group were recorded. The minimum lethal dose of the virus was considered to be the highest dilution which produced fixed-virus rabies in 50 per cent of the infected control mice. The end-point of immunity was the lowest dilution of the test virus measured in terms of minimum lethal doses in which at least 50 per cent of the infected mice escaped fixed-virus rabies.

The calculations were made in accordance with the method suggested by Reed and Muench (1937).

MOUSE-TEST DETERMINATIONS OF VARIOUS VACCINES.

Semple's vaccine.—Several determinations have been made on random samples, extending over two years, on the 5 per cent Semple's antirabic vaccine which

is manufactured at this Institute. The results obtained are summarized in Table I:—

TABLE I.

Showing results of mouse test on random samples of 5 per cent Semple's vaccine prepared at the Haffkine Institute.

Serial number.	Batch number.	Date.	50 per cent end-point.	M.l.d. of virus.	M.l.d. protection of vaccine.
1	323/46	8-6-46	$10^{-2.5}$	10^7	$10^{4.5} = 31,620$
2	820/46	4-2-47	10^{-3}	$+10^8$	$+10^5 = +100,000$
3	225/47	1-5-47	$10^{-2.2}$	$10^{7.15}$	$10^{4.95} = 89,130$
4	479/47	8-7-47	$10^{-2.6}$	$10^{7.2}$	$10^{4.6} = 39,810$
5	1145/47	16-12-47	$10^{-3.5}$	10^8	$10^{4.5} = 31,620$
6	1033/47	20-4-48	$10^{-4.2}$	$+10^9$	$+10^{4.8} = +63,100$

The data appear to be sufficient to indicate that (a) different brews of our vaccine during 1946-47 showed values of immunity protection in mice of a high order and (b) the figures obtained in all the above determinations were remarkably uniform. Further batches will be tested as a routine procedure.

CHLOROFORMED AND PHENOLIZED VACCINE.

Using the same technique chloroform- and phenol-treated vaccines prepared from the same rabbit's brain were also tested for purposes of comparison. The rabbit was inoculated with Paris strain fixed virus. On appearance of signs of paralysis the brain was dissected, emulsified and equally divided into two parts. One part was made up with 0.5 per cent phenol and the other with 2 per cent chloroform. The finished 5-per cent brain tissue products thus obtained were tested. The results of the mouse test are shown in Table II:—

TABLE II.

Immunizing values of phenolized and chloroform-treated 5-per cent rabbit-brain vaccine.

Vaccines used.	50 per cent end-point.	M.l.d. of virus.	M.l.d. protection of vaccine.
Phenolized 5-per cent rabbit-brain vaccine.	$10^{-3.25}$	$10^{7.8}$	$10^{4.55} = 31,990$
Chloroform-treated 5-per cent rabbit-brain vaccine.	10^{-3}	$10^{7.8}$	$10^{4.8} = 63,100$

This experiment, although only one, is recorded to substantiate experience of other workers in that the chloroform-treated vaccines were better immunizing agents than phenolized vaccines.

OTHER VACCINES.

Since 1938, all the Pasteur Institutes in India have been using a uniform technique for preparation of rabies prophylaxis. They all employ Paris strain fixed virus. Their manufactures were obtained and tested along with our own product by the method described above. The results are set out in Table III :—

TABLE III.

Immunizing potency of rabies vaccines from different Pasteur Institutes in India.

Source and type of vaccine.	50 per cent end-point.	M.I.d. of virus.	M.I.d. protection of vaccine.
Pasteur Institute, Coonoor, 5 per cent Semple's vaccine.	10^{-1}	$10^{6.2}$	$10^{5.2} = 301,000$
Pasteur Institute, Shillong, 5 per cent Semple's vaccine.	$10^{-1.2}$	$10^{6.2}$	$10^5 = 100,000$
Central Research Institute, Kasauli, 5 per cent Semple's vaccine.	10^{-1}	$10^{6.2}$	$10^{5.2} = 301,000$
Pasteur Institute, Calcutta, 2 per cent Semple's vaccine.	$10^{-2.4}$	$10^{6.2}$	$10^{3.8} = 9,031$
Haffkine Institute, Bombay, 5 per cent Semple's vaccine.	10^{-1}	$10^{6.2}$	$10^{5.2} = 301,000$

It would be observed that the potency values of vaccines are about the same in all cases except in the case of Calcutta vaccine which is a 2 per cent Semple's vaccine.

The experiment was carried out earlier in our work. Six-week old mice weighing 20 g. to 25 g. were used. This probably was responsible for the lower m.i.d. values obtained for the test virus as compared with values obtained in later work from the same strain. The higher m.i.d. protection values for the vaccines would possibly result for the same reason.

CONCLUSIONS.

1. In our hands Habel's modification of Webster's mouse test has worked satisfactorily with Haffkine Institute inbred white mice. We recommend its use as a routine test in manufacture of rabies prophylaxis to all Pasteur Institutes in India.

2. The mouse-test m.l.d. protection values of the vaccine do reflect satisfactorily on the potency of the phenolized 5 per cent Semple's vaccines as prepared in India.

Our thanks are due to the Director, Haffkine Institute, Bombay, for his interest and to the staff of the Department of Virus Diseases for their technical assistance.

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NOTICE.

The following has been received for announcement :—

Editor. I.J.M.R.

15th September, 1948.

COMMONWEALTH AND EMPIRE HEALTH & TUBERCULOSIS CONFERENCE, 1949.

**UNDER THE AUSPICES OF THE NATIONAL ASSOCIATION FOR
THE PREVENTION OF TUBERCULOSIS, LONDON.**

THE Jubilee of the NAPT will be celebrated during 1949, and one of the principal functions in this connection will be the Commonwealth and Empire Health & Tuberculosis Conference at the Central Hall, Westminster, London, S.W.1, on 5th, 6th and 7th July, 1949. The programme will include the following subjects :—

TUBERCULOSIS AS A WORLD PROBLEM.

MODERN TREATMENT AND ITS RESULTS, INCLUDING P.A.S. AND STREPTOMYCIN.

MASS X-RAY METHODS : THEIR EVALUATION AND SCOPE.

REGIONAL COUNTY TUBERCULOSIS SCHEMES.

ORGANIZATION OF COMPREHENSIVE TUBERCULOSIS SCHEMES IN BRITISH COLONIES.

A SOCIAL QUESTION (subject to be chosen later).

TUBERCULOSIS AMONG NURSES AND STUDENTS.

THE ERADICATION OF BOVINE TUBERCULOSIS.

A fuller programme will be issued in the autumn.

NAPT Prize for Essay on Colonial Tuberculosis.—A prize of one hundred guineas will be awarded for an Essay on THE CONTROL OF TUBERCULOSIS IN A BRITISH COLONY. The competition is open to doctors of either sex in the British Colonial Medical Service who are not more than ten years' or less than five years' medical standing, of which at least three years have been spent overseas in a medical capacity.

Competitors should describe their own proposals for a practical scheme for the clinical, social and administrative control of tuberculosis, either in the British Colonies as a whole, or in one or more of them separately.

2. The mouse-test m.l.d. protection values of the vaccine do reflect satisfactorily on the potency of the phenolized 5 per cent Semple's vaccines as prepared in India.

Our thanks are due to the Director, Haffkine Institute, Bombay, for his interest and to the staff of the Department of Virus Diseases for their technical assistance.

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SCRUB TYPHUS SUBSEQUENT TO 'FULTON' VACCINE AND INVESTIGATION OF THE INFECTED SITE.

BY

H. C. BROWNING,
M.R.C. Field Typhus Team.

AND

S. L. KALRA,
G.H.Q. (I) Field Typhus Research Team.

[Received for publication, May 23, 1948.]

A SUSPECT localized infected site at Palel, 33·4 miles from Imphal on Imphal-Tamu Road in Manipur State, was investigated from September to November 1945. During the investigation a moderately severe case of scrub typhus occurred in a member of the first named team working at this site. The results of the field and laboratory epidemiological investigation are reported.

INCIDENCE OF CASES AT THE SITE.

Two hundred and sixty-one personnel of a Pioneer Force camped from July to 23rd September.

There were 17 cases with one death and one case in the personnel of the Typhus Team who worked at the site. The first case occurred on 17th July and the last on 12th October. Seven cases had onset in July, 5 in August, 4 in September and 1 in October.

EXPERIMENTAL EPIDEMIOLOGY.

Vegetation.—The camp was at an elevation of 3,400 feet above sea-level, situated on an outlier on level with the road, approximately 100 yards long and 30 yards wide. It was cleared and occupied from virgin scrub. The ground dropped sharply on all sides into scrub jungle. On the south side of the outlier the slope became gradual 150 feet below the camp. Here was situated a pond which was used for bathing and where the infections were supposed to have been acquired. The investigation was mainly confined to the pond and its surroundings.

The scrub jungle was secondary to felling or burning of the natural jungle and typical of the whole Imphal area. It consisted of rank grass from 4 to 20 feet

high. The dominant species was *Neyraudia reynaudiana* (Kunth) Keng, called *Kyu-na* in Burmese. It is moisture-loving and fairly common in damp places. The sub-dominants were *Arundinella nepalensis* Trin.; *Cymbopogon khasianus* Stapf. Ex. Bor.; *Phragmites karka* Trin. Besides grasses, stunted trees, mainly species of oak, occurred irregularly in this area.

In the experimental area four smaller areas were used. The first of these was the pond border. The latter was roughly 25 yards by 15 yards but was not permanent, drying up twice during the period of work. The second, damp scrub, was main part of the area where the soil beneath the grass was moist and sometimes moss covered. The third, dry scrub, was on a hillock adjacent to the pond and the soil here was dry. All sites were within 50 yards of each other. The soil within a foot radius of the trees was dryer and sparser in vegetation and was considered the fourth site.

Weather during period of work.—During September it was almost always raining or overcast in the mornings and hot and sunny in the afternoons, with a shade temperature of 85°F. to 95°F. In mid-October there was heavy rain. In November there was no rain but mist covered the area from nightfall till 10:00 or 11:00 hours. The maximum and minimum shade temperatures in the grass of the scrub were 68°F. to 79°F. and 43°F. to 46°F. in November.

Experimental infestation.—From 3rd to 14th September ten cages, with quarter-inch wire-mesh bottoms covering 6 square feet and each containing 5 mice were exposed continuously, scattered over all the sites. On the 11th a *Trombiculid* larva was found wandering on a mouse and one attached to the ear of the mouse in the damp scrub. On this day two such larvæ were found on the boots of an assistant working in the area though none occurred on four others. On the 7th day another free mite was found on a sick mouse in a damp scrub cage and on the 11th another mouse in this area had an attached larva in its ear which remained *in situ* for four days. As the maximum chance of infection was required, none of these mites were removed for identification. So far as could be seen under a hand lens, they had the coloration and appearance of *Trombiculid* larvæ.

All animals were examined daily and before and after exposure for *Ectoparasites*. Apart from those noted above one tick was found on the ear of a mouse in the damp scrub. All the *Ectoparasites* were recovered from the damp-scrub site only.

After withdrawal all mice were observed for a month and sick or dying animals passaged by brain emulsion into fresh mice intraperitoneally. One mouse with an attached larva died after four weeks and itself was positive for rickettsia in spleen smears, but three generations of passaging were negative. It was later observed with another strain (p1) from this area that the rickettsia appeared for the first time in the 4th passage.

Later, wild rats had been found a far more attractive bait for *Trombiculid* larvæ than mice and were exposed at these sites. Each was placed out in a quarter-inch wire-mesh cage 8" by 6" by 4" for 24 hours, withdrawn and examined and replaced 24 hours later. Examination was carried out under bromethol anaesthesia.

The rats were *Rattus rattus* caught in site where infection had not been found.

TABLE I.

Individual Trombiculid larvæ infesting wild-rats exposed for 24 hours.

Site.	Date of exposure and individual number of infesting mites (September).																							
	1	2	3	4	5	6	7	8	9	13	14	15	16	17	18	19	20	22	23	24	25	26		
Pond edge	3	45	6	93	0	0	2	...	0	...	0	...	0	0	0	0	0	...	
	2	6	1	0	0	0	0	0	0	...	0	...	0	0	0	...	
	0	0	24	0	1	...	0	...	0	0	0	...	0	...	
	4	...	0	1	5	0	...	
	2	0	
Damp scrub	1	0	1	0	83	2	0	0	0	1	0	...	0	...	0	0	0	...	
	2	...	0	0	0	0	0	0	0	...	0	...	0	...	
	0	...	1	...	0	0	0	
	53	...	13	
	5	
Tree bushes	4	...	0	...	1	
	0	2	3	0	1	70	0	...	0	0	2	1	0	0	0	0	0	...	0	0	0	0	...	
	3	2	3	...	5	0	2	...	0	0	4	1	1	...	0	...	0	0	0	...	
Dry scrub	2	...	0	0	0	...	0	0	
	7	0	...	0	...	4	3	4	0	...	0	1	1	...	2	0	0	0	0	...	
	8	...	0	...	0	1	0	0	...	1	0	...	0	0	0	
	15	0	14	...	2	1	

TABLE II.

Average infestation of rats and chance of positive infestation.

Period.	POND EDGE.		DAMP SCRUB.		TREE BASES.		DRY SCRUB.	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Average infestation per 100 rats.	1,240	37	1,025	18	650	55	275	200
Percentage chance of any infestation.	66.7	16.7	62.5	11.7	64.3	31.8	50.0	52.4

Considering all figures the distribution of free larvæ was erratic. The highest catches were in damper soil. The tree bases were all in the damp-scrub area yet infestation was lower, possibly because less grass offers less cover and less protection from sun and drying. Even so the larvæ cannot have a great range for no tree base was more than 3 yards from fully developed scrub and the visible area influenced by these stunted trees around their bases was not more than a foot in radius.

On the other hand the possibility of any infestation was almost identical for all sites. Similar results were obtained in rats trapped at Mandalay during a period of declining mite population. The number of *Trombiculid* mites on rats decreased by one-third but the percentage of infested rats remained the same. Presumably one mite is sufficient for infection for records of more than one eschar in human patients are rare; so chance of infection will be more related to mite infection than to mite numbers in any area.

By the second period of exposure there had been a marked drop in infestation rates, except for the dry-scrub area which was almost constant. The weather had remained the same apart from gradual temperature drop through the month. Either the latter passed through a critical point between 7th and 13th November or all available larvæ in the areas used had been provided with hosts. Yet cages had been moved up to three yards from original sites occasionally throughout the experiment. For larvæ to have all been removed on the rats would infer a larger range of movement than seems likely from results with the tree bases. From 25th November cages were again moved to at least 10 yards but remaining in identical types of area. Catches remained *nil* or negligible. Probably both removal of available larvæ by live bait and disappearance of free larvæ due to cold were responsible for these results.

Area soils.—From each area soil sample was taken by removing the top 1½", mixing and sieving out free vegetation by 16 per inch mesh. The moisture and organic matter content in Table III are for 10-gramme samples. An empirical estimation of particle composition was made by mixing 25-gramme samples of each with 100 c.c. of water and allowing to stand for 24 hours in a graduated cylinder. All samples had a pH of less than 6.8, the lowest value which could be determined with available field apparatus.

TABLE III.

Some constituents of area soils.

Constituents of area soil.			Pond edge.	Damp scrub.	Tree bases.	Dry scrub.
Water content	3.01	2.80	2.24	2.51
Organic content	0.68	0.70	0.81	0.94
Floating organic matter	+	++	++++	++++
Coarse particles	10	17	18	18
Fine particles	18	14	14	14

The differences in the above constituents are not marked enough to explain differences in catches.

Reservoir and vector species.—The commonest species of rat was *Rattus rattus* variety near *R. rattus brunneusculus*. More rarely *Rattus manipulus* and *Hydromys humei* Thomas, as well as field mouse were caught. Tree shrew (*Tupaia belangeri belangeri* Wagner) was also present in the area. All showed infestation with *Trombiculid* larvae.

The following species of mites were found on rats exposed experimentally or trapped at the site:—

Trombicula deliensis Walch; *Walchia glabrum*; *Schöngastiella ligula*; *Neoschöngastia cockingsi*; *Paraschöngastia*; *Trombicula rebri*; *Walchia* (species not known).

Ticks. *Hæmaphysalis leechi* var. *indica* infesting a *R. rattus*.

Six soil samples were taken from the area. One consisting of humus from a tree crotch showed 9 nymphal and two larval *T. deliensis*. A second sample taken from damp scrub showed two *T. deliensis* adults. The remaining samples, three from the pond area and one from the damp scrub, were negative.

Infection in vector species and reservoir species.—Seven strains of *R. tsutsugamushi* were recovered from three different types of rodents and one insectivore; four strains from pools of mixed species of *Trombiculid* mites; one from a patient. One strain of tick typhus was recovered from ticks.

- (a) *R. rattus* variety near *R. rattus brunneusculus*, 14 examined; one strain recovered from brain tissue and 3 from blood. The presence of rickettsæmia was evidence of active infection. for in another experiment it was found that rickettsæmia persisted in the blood of these rats for a maximum period of 75 days only.

- (b) *Hydromys humei* Thomas; 4 examined in pools of two each; one strain recovered.
- (c) Tree shrew (*Tupaia belangeri belangeri* Wagner); one examined and found infected.
- (d) Field mice; 12 examined; one found infected.
- (e) Twenty batches of mites collected from rats were examined and four strains were recovered. The species composition of the positive pools was as follows:—
 - (i) *T. deliensis* and *Schöngastia ligula*.
 - (ii) *T. deliensis* Walchia (species not known); *Parashöngastia* (species not known); *Schöngastia ligula*; *T. webri*.
 - (iii) *T. deliensis* and *Neoshöngastia*.
 - (iv) *T. deliensis* and others (not identified).
- (f) A strain was isolated from the blood of the patient (H. C. B.). The case is described below.
- (g) A strain of rickettsia was isolated from the ticks *Hæmaphysalis leechi* var. *indica*. It was immunologically identical with Rocky Mountain spotted fever but its virulence for guinea-pigs was very low.

Case of scrub typhus subsequent to 'Fulton' vaccine.

History.—The patient H. C. B., aged 33, received three 1-c.c. subcutaneous doses of vaccine at weekly intervals between 24th April and 8th May, 1945, and was exposed to infection from 26th June, 1945. Symptoms of benign tertian malaria were present from 21st to 26th August inclusive. There had been no other medical history for at least two years.

The area was visited on 5th, 6th and 9th September. On each visit a period of four hours was spent in the scrub, partly in active movement and partly in stationary observation. Almost every day there was rain in the morning and bright sunny conditions in the afternoon. No protective measures were taken against infection. Eight other vaccinated personnel and three unvaccinated paid one or two visits to the area and one vaccinated in early June spent 12 four-hour periods in the scrub between 5th and 19th September under similar weather conditions without protection and without developing infection. The patient had always within two hours of leaving the site bathed with soap and warm water scrubbing particularly of the thigh area where an eschar appeared. Either such treatment does not remove attached mites; or mite attachment for less than six hours is enough to convey infection or mites remain in clothing from two to six hours and then can attach. In another member of the team a mite was seen attached after bathing. Next day the portion of his skin with the still attached mite was removed. Serial sections of both revealed a localized area of inflammation in the skin at the site of the attachment of the mite but the internal tissues of the mite were degenerated. The mite was probably killed by bathing but not detached. In such a case if the mite is infected the damage will be done before

the mite is killed; for injection of the saliva is the first action of the mite on attachment.

Following the exposure of 9th September the patient was engaged in laboratory work and neither visited suspect sites nor left the long 'civilized' area of the Typhus Unit Camp until 2 days before the onset of the disease. At this time he spent very short periods in the scrub of the camp area. The fact that no scrub-typhus cases occurred in the Unit Camp for at least six months, very strongly indicated that the infection was acquired at the Pioneer Camp area.

As the first exposure was on the 5th and the last on the 9th while the symptoms started on the 19th, the incubation period lies between 10 and 14 days.

Clinical.

(a) *Prodromal.*—Late on 19th September there was mild aching of the legs and the patient noticed a lesion on the thigh consisting of a pus-filled area surrounded by a zone of inflammation. The following day temperature began to rise and this (20th September) has been taken as the first day of fever. Malaria relapse was suspected and quinine-mepacrine treatment commenced although blood films prior to the first dose showed no parasitaemia. Steadily rising temperature, full development of the eschar and tender enlarged lymph glands resulted in a clinical diagnosis of scrub typhus on the third day when the patient was hospitalized.

(b) *1st to 3rd day.*—Steady rise of temperature to 104°F.; initial rise of pulse rate to 96 with fall to 80; eschar on left thigh with inguinal and axillary lymph glands enlarged and tender; post-cervical glands just palpable; spleen palpable, firm and tender; face flushed; malaise, dull headache and insomnia.

(c) *4th to 5th day.*—Temperature fluctuating between 102°F. and 105°F.; pulse rate increasing to 92; spleen one finger; liver not palpable, no rash or tremor; headache gone; patient drowsy but coherent. Apparently improved on the 5th day and sitting up reading.

(d) *6th to 12th day.*—Condition deteriorating, with increasing drowsiness. Temperature fluctuating between 101°F. and 105°F.; pulse rate over 100 and crossing temperature figure from 8th day. On the 8th, 9th and 10th days there were frequent and persistent attacks of hiccoughs, eventually physiologically and psychologically initiated by fluid intake, thus adversely affecting the latter.

From the 8th day there was increasing delirium; and all its features bore an obvious relation to the patient's work and personal history. During this period there developed hand tremors, ptosis of lower jaw, incapacity to focus the eyes on a specific object for any length of time and incoherence of speech. With the possible exception of the latter these symptoms were probably due to generalized muscular weakness and were not neurological.

(e) *12th to 16th day.*—During the 12th, 13th and 14th days the temperature fell by slow lysis, with great improvement in the general condition. Delirium

slowly subsided though the patient remained irrational, particularly at night, until the 16th day. Tremors of the hand were marked.

(f) *16th to 20th day.*—The drowsy apathy of quiescent periods gave way to completely normal cerebration. The lymphatic glands regressed but the spleen remained one finger below the costal margin. The patient ended this period well but very weak and wasted.

(g) *20th to 28th day.*—Liver and spleen no longer palpable; no evidence of myocarditis; slight hand tremors more apparent in the mornings after rising and in the late afternoons. Discharged to unit for convalescence on 28th day (18th October).

(h) *General.*—From the time when the patient was capable of appreciating it (16th day) there was deafness in both ears, more markedly in the left. This persisted till the 20th day when some improvement was noted. Normal hearing returned in the next two days. That it was due to quinine is unlikely, for administration of this ceased on the third day, and in the patient's malarial attack, deafness from this drug was of a different type and disappeared four days after 30 grains had been given for five days.

From the 20th day when the patient first got out of bed paræsthesia of the soles of the feet, and especially of the toes, was noticed. The condition persisted into convalescence with diminishing intensity. Hyperæsthesia of the teeth occurred and persisted in the same fashion. A feeling of unsteadiness in coming down steps remained for a long time. No rash developed. Cough was not present. There were neither any chest symptoms nor complications that are commonly described. Constipation, especially in the first week and to an appreciable extent in the second, was marked and troublesome.

(i) *Temperature and pulse rate.*—The duration of fever was 13 to 14 days. The course of fever, with rapid temperature rise to 103°F. to 105°F. and daily fluctuations of 2½°F., fell by lysis between the 12th and 14th days. Minor terminal rises on the 15th and 18th days are also typical.

After an initial drop from a relatively high pulse the rate steadily rose and remained above the temperature line till the 9th day when it fell along with deferescence. It was slower than would be expected for the degree of fever in the first week. There was a distinct rise in the pulse rate from the time of leaving hospital, presumably due to a more active life in convalescence.

The treatment was symptomatic.

PATHOLOGY.

Eschar.—The day before onset of fever, an eschar was found in the centre of the inner left thigh. It had an inflamed margin 3 mm. wide surrounding a pus-filled centre 9 mm. by 3 mm. The inflamed area was slightly raised but the pus area flat, and the two were quite sharply delimited from each other. Inflammation was at its maximum on the 3rd day but the lesion was never painful or irritating. A dark scab, developing centripetally, covered the entire pus area by the 5th day; it was slightly indented. Inflammation was now disappearing. From this time

onwards there were no changes beyond the gradual resolution of the necrotic portion and early disappearance of inflammation. On the 17th day the area was excised for serial sections. The necrotic portion was about to slough and the skin had completely regenerated beneath it except at the central point. No trace of stylostome or other evidence of mite attachment was found; no rickettsia were seen in the sections.

Total and differential white blood counts.—Total counts showed a slight degree of leucopænia in the early stage of the disease followed by a steady rise reaching a peak (11th day) as defervescence was about to occur. After this there was return to a normal count. Eosinophils were depressed for the first four weeks. There was an increase in azurophilic granules in the lymphocytes which persisted through convalescence.

Weil-Felix reaction and complement-fixation test.—Widal test and Weil-Felix reactions were consistently negative to all but *proteus* OXK. A diagnostic titre was first obtained on the 14th day—1,260; it rose till the 24th day—163,840. From this time there was a steady but slower decrease into convalescence.

Serum taken on the 18th day and tested in the Laboratories of the National Institute of Health, Bethesda, Md., U.S.A., by the courtesy of Dr. Topping, gave the following complement-fixation titres:—

Against Gilliam antigen—positive at 1:32.

Against Karp antigen—positive at 1:512 (end-point not reached).

Isolation of rickettsia.—Blood clot from 5 c.c. blood withdrawn on the 3rd day was ground up in saline and inoculated intraperitoneally into two mice. One animal died on the third day and re-passage of brain suspension into two further animals resulted in death of both from bacterial contamination. The other original mouse was killed on the 16th day after inoculation, when it showed copious intraperitoneal and intrapleural fluid, but no rickettsia or any other organism. Re-passage of brain suspension from this animal to four others gave the following results:—

Mouse 1: Died after 9 days, negative findings.

Mouse 2: Killed after 14 days, copious intrapleural and peritoneal fluid, no organism seen, re-passaged into two mice.

Mouse 3: Killed after 23 days, findings same as in No. 2 above.

Mouse 4: Survived 40 days' observation.

Two mice in the third generation, killed after 15 and 24 days, negative findings. The former passaged into 3 mice. Two died after 14 days and one after 15 days. Pleural and peritoneal exudate also typical rickettsia present in all the mice.

From the 4th to the 9th passage the results in mice were consistent. From the 10th passage there was again loss of virulence. The average period of survival in a total of 22 mice dying normally of infection was 12.2 days, more or less the same as with other strains from this site. This strain is referred to as 'pl' strain. All the other strains from this site showed visible rickettsia and typical post-mortem

findings from the very beginning. The results of 'D2,' one of the mite strains from this site, and 'pl' strain are tabulated below:—

Passage.	'P1' STRAIN.			Passage.	MITE STRAIN 'B2'.		
	R	Pe	Pl		R	Pe	Pl
1. K 16 ...	—	+++	+	1. D 16 ...	+	+++	+++
2. { D 9 ...	—	—	—	2. { D 14 ...	+	+++	+
2. { K 14 ...	—	+++	+	2. { D 15 ...	+	+++	++++
2. { K 23 ...	—	+++	+	2. { D 15 ...	++	+	+++
Survived.							
3. { K 24 ...	—	—	—	3. { D 14 ...	++	+++	+++
3. { K 15 ...	—	—	—	3. { D 14 ...	+++	+++	+++
				3. { D 15 ...	++	+	++
4. { D 14 ...	+++	++	+	4. { D 10 ...	++	+	+++
4. { D 14 ...	+	+	+	4. { D 11 ...	++	+	+++
4. { D 15 ...	++	+	+	4. { D 11 ...	++	+	+++

D—died; K—killed; R—rickettsia; Pe—peritoneal exudate; Pl—pleural exudate; the numerals indicate the day.

Immunity studies.

'P1' strain from patient H. C. B.—*M. rhesus* monkeys when inoculated with scrub-typhus strains developed local skin reaction at the site of inoculation; fever, rickettsæmia, lymphopænia, increase in azurophilic granules in the lymphocytes and a rise in OXK agglutinins.

Two monkeys that had previously been inoculated with 'K' mite strain from Tamu were re-inoculated with 'pl' strain. Both of them did not show any of the above-mentioned responses.

A *M. assamensis* was captured locally from an infected site in Imphal and was infested with *Trombiculid* mites on capture. It was inoculated with 'pl' strain. There was neither any local reaction or fever nor rise in OXK agglutinins, but rickettsia were recovered from its blood on the 6th day. The response was a near complete immunity.

Mice.—Eight white mice were immunized by subcutaneous injection of 'pl' strain and 40 days later were inoculated intraperitoneally with 'b1' mite strain from Imphal. The challenge dose was 20 times greater than the immunizing dose.

Four mice survived an observation period of 40 days. Another 6 mice similarly immunized by another mite strain 't2' from this site were all protected against 'p1' strain.

Two other strains from this site 'D2' from mites and 'B2' from rat showed complete two-way immunity against a patient, mite and rat strain isolated from other parts of Imphal area.

'W2' isolated from tree shrew.—A *M. rhesus* inoculated with 'B2' rat strain from this site developed local reaction, fever, rickettsæmia and OXK agglutinins only in a titre of 1: 320 on the 54th day. On re-inoculation with 'W2' strain there was no local skin reaction, no rickettsæmia but a short fever was present. *Proteus* agglutinins developed for OX2 only instead of OXK in a titre of 1: 620 on the 36th day and 1: 1,280 on the 58th day. Except the difference in *proteus* agglutinins the reaction was that of a partial immune animal.

Four mice were immunized with this strain. None of them survived a challenge dose of 'b1' mite strain from Tamu.

In morphology and reaction in mice the strain was typical of *R. tsutsugamushi* strains studied so far.

DISCUSSION.

Besides the above results tests on rabbit's eye with strains isolated from Imphal, Tamu, Paungde in Burma, Ceylon and Kumaon Hills in India proved that there was more or less complete immunity for local strains from different sources, but only a partial immunity for strains from distant areas. Similarly, the study of Imphal-Burma strains in *M. rhesus* monkeys showed that dermal immunity was solid but generalized immunity was not always complete even on a third inoculation. The complement-fixation test with the serum of patient H. C. B. gave significantly different titres with antigens prepared from different strains. This is in line with immunological differences observed in different strains by other means.

Clinically, there are variations in particular symptoms from place to place although the symptoms in any one area are generally consistent. The mortality again varies a great deal in different parts. Amongst Indians in Addu Atoll it was 2.4 per cent; Tamu 11.4 per cent and at Meiktila nil.

These differences in strains from distant parts will render it difficult to rightly assess the value of a single-strain vaccine used in different countries. One would rather recommend the use of a polyvalent vaccine for scrub typhus.

In connection with human infections Hatori (1919) states that the native population are less susceptible than newcomers in endemic districts. We observed the same thing in Addu Atoll (Maldiv Islands) and Ceylon. Hatori (*loc. cit.*) also observed re-infections in two cases after an interval of several months and one after about two years. He adds that a single attack gives at least relative immunity as second ones are milder. Nagayo (1923) considers that a certain degree of immunity results from infection but he found that fresh infection could occur in the same place. Lewthwaite (1930) is the only author who suggests that complete immunity is conferred by a single attack, at least for one year. He found that, out of 28 prisoners exposed to infections at the Oil Palm Estate near Kuala Lumpur

Institute (Rao and Dogra, 1948). Immunizing value of a canine vaccine prepared in this laboratory was assayed. The results of the experiments are reported in this communication.

MATERIALS AND METHOD.

A 25 per cent phenolized sheep-brain antirabic vaccine was prepared on lines suggested by Savor (1946), one of the workers from Institute of Medical Research, Kuala Lumpur, who came and worked at this Institute during the Second World War. The Paris strain fixed virus used for routine manufacture of 5 per cent Semple's sheep-brain vaccine for human use was used. On appearance of signs of paralysis the sheep was de-capitated, brain dissected and to every 100 g. of brain tissues 160 c.c. of saline with 1.25 per cent carbolic was added and incubated at 37°C. for 24 hours. Next day 240 c.c. of glycerine was added to make 25 per cent brain-tissue emulsion. This emulsion was then incubated at 37°C. for another 6 days, tested for sterility and ampouled.

For purposes of comparison, a sample of 5 per cent sheep-brain phenolized Semple's vaccine was taken at random and tested along with the above canine vaccine.

Haffkine Institute inbred white mice, about four weeks old, weighing 16 g. to 18 g., were used and Habel's technique followed. About 40 mice were used in each experiment. The canine vaccine was given in one dose of 0.3 c.c. after diluting it 1 in 10 to reduce its carbolic content. The Semple's vaccine also diluted 1 in 10 was given in six doses of 0.25 c.c. each on alternate days. All injections of vaccine were given intraperitoneally and the test-virus challenge dose intracerebrally.

COMPARATIVE ASSAY OF A CANINE AND SEMPLE'S VACCINE.

In the first instance, two experiments were conducted. The results are shown in Table I:—

TABLE I.

Showing the immunizing value of a 25 per cent canine and 5 per cent Semple's phenolized vaccine.

			50 per cent end-point of immunized mice.	M.l.d. of test virus.	M.l.d. protection of vaccine.
<i>1st experiment :</i>					
Semple's vaccine	$10^{-2.6}$	$10^{7.2}$	$10^{4.6} = 39,810$
Canine vaccine	Nil.	„	0
<i>2nd experiment :</i>					
Semple's vaccine	$10^{-4.2}$	10^9	$10^{4.8} = 63,100$
Canine vaccine	Nil.	„	0

These experiments showed complete absence of any immunizing value of the canine vaccine under test, whereas the Semple's vaccine showed a high protection value.

The vaccines, namely, Semple's and canine, in the two experiments were prepared from different sheep brains. A third experiment was, therefore, carried out. In order to exclude the variations of virus content in different sheep's brain, one sheep's brain was divided into two equal portions. One portion was made up in the usual way into 5 per cent Semple's vaccine and the other into a 25 per cent canine vaccine. Simultaneously, three batches of animals were put up along with one control batch. This was done to observe the extent of experimental variations that may occur in the mouse test in our hands, using a large number of animals at one time. Total quantity of brain tissue given with Semple's vaccine and the canine vaccine was the same except that the former was given in six doses and the latter in one dose—all intraperitoneally. In each group and for each dilution of the test virus eight Haffkine Institute inbred white mice were used. The results are set out in Table II:—

TABLE II.

Showing the immunizing value of 5 per cent Semple's vaccine and a 25 per cent canine phenolized vaccine prepared from the same sheep brain.

			50 per cent end-point of immunized mice.	M.l.d. of test virus.	M.l.d. protection of vaccine.
<i>Batch A :</i>					
Semple's vaccine	$10^{-3.79}$	$10^{7.16}$	$10^{3.37} = 2,344$
Canine vaccine	0	"	0
<i>Batch B :</i>					
Semple's vaccine	$10^{-3.89}$	$10^{7.16}$	$10^{3.27} = 1,862$
Canine vaccine	0	"	0
<i>Batch C :</i>					
Semple's vaccine	$10^{-4.13}$	$10^{7.16}$	$10^{3.03} = 1,072$
Canine vaccine	0	"	0

It would be observed that in these three batches of animals results were fairly uniform and showed no marked experimental variation and that the canine vaccine under test showed no protective value whatsoever.

As the necessity of a suitable canine vaccine is very keenly felt, work has been undertaken to prepare a canine vaccine which would give the necessary protection to dogs with one injection. This work is now in progress.

SUMMARY.

1. Using Habel's technique with Haffkine Institute inbred white mice fairly uniformly consistent results were obtained in the experiments reported.

2. The 25 per cent phenolized sheep-brain canine vaccine prepared in the way described by Dr. S. R. Savoor showed no protective powers, whereas the 5 per cent Semple's vaccine did give results which were satisfactory.

The author wishes to acknowledge with thanks the interest shown by the Director, Haffkine Institute, Bombay, in this work and to the staff of the Department of Virus Diseases for their technical assistance.

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A MYOTROPHIC INDEX.

BY

G. M. KURULKAR.

(From the Seth G. S. Medical College, Parel, Bombay.)

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INTRODUCTION.

AN efficient anthropometric measure of an individual's nutritional status is a great necessity for workers in the field of nutritional researches. During the last 100 years, a number of anthropometric indices have been proposed by various workers for the purpose of estimation of individual's nutritional condition. Most of them have been thoroughly tested and rejected by several critics for one reason or other. Recently, when the inquiry 'Basic diet in India' was being conducted in the G. S. Medical College, Bombay, an occasion arose for testing the efficiency of these nutritional indices. None of the available indices were found to be reliable.

Having had the personal experience of these trials, the author thought of experimenting with some new anthropometric formulae which may possibly give better results.

To express the state of nutrition in terms of physical measurements has been a difficult matter. Most of the early somatometric indices (see *Appendix*) suggested for the estimation of nutritional status were based on the consideration of body-weight as related to stature, or stature squared or cubed (Buffon, Quetelet, Rohrer). Others divided the cube root of weight by height (Livi), or the cube of height was divided by weight (Bobbit). The evolution of these formulae indicates that the conception of a good nutritional status in the minds of these workers was in terms of weight as related in different ways to height. All these attempts were not satisfactory. Bernhard (1886, quoted by Sankaran, 1940) introduced a chest measurement in addition to height and weight. In his opinion, as his formula indicates:—

$$\frac{\text{Height in cm.} \times \text{Chest measurement in cm.}}{240} = \text{Weight in kg.}$$

a good nutritional status is discernible in terms of chest-girth-and-height as related to weight. Pignet (1901, quoted by Sankaran, *loc. cit.*) also included chest measurement in his formula. All these indices were declared unsatisfactory by critics.

Tuxford (1917) thought of giving an age correction to weight : height ratio. Others, such as Broca and von Noorden, suggested some constants to the weight : height ratios.

Pirquet (1920, quoted by Sankaran, *loc. cit.*) suggested 'Pelidici'. This index is nearly empirical.

It will be observed that so far body-weight in relation to height was a necessary factor in the index and a chest measurement only a secondary necessity. All these indices were proved to be not reliable, and owing to these failures, there developed a revulsion to use the body-weight in an index. Franzen proposed the A.C.H. index in which the factor of body-weight has been scrupulously left out. The conception underlying this index is that for an estimation of nutritional status quantity of muscle and fat should be measured directly and that it should be related to the skeletal build. A.C.H. index is so called because it is an index of arm, chest and hip. This index seemed, at first sight, to offer considerable possibilities. Extensive trials were given in this country as well as elsewhere and the index did not prove very satisfactory. Jones (1938) concludes that indices involving chest and hip measurement do not show any particular advantage over those based on weight and height alone in selecting children who are judged clinically to be under-nourished.

As revulsion to weight for being used as a factor in a nutritional index slowly grew less, there developed a tendency 'to find rough measures of volume of the body' or take a product of three linear dimensions which may reasonably be supposed to be proportional to it (Karl Pearson). 'In other words, the length, width and antero-posterior thickness of an infant should be measured.' 'Of the three such measures,' Sankaran (*loc. cit.*) says, 'the first two (stature and pelvic-width) are capable of accurate measure, while the antero-posterior chest measure is capable of great fluctuations due to the respiratory function.' 'Karl Pearson', Sankaran adds, 'considers the average body-density as an excellent index and suggests as an ideal, the determination of volume of an infant by immersion. But this is not practicable.' Pearl (1940) in his critical survey of the somatometric indices remarks that, of all the old indices showing the direct relationship of the body-weight to stature, the ratio $\frac{\text{body-weight}}{\text{stature}}$ still remains the most satisfactory of all. Further, for the guidance of future workers, he formulates some desiderata for an index of body-build.

1. Its numerical values should march in a unidirectional manner with commonsense understanding and interpretation of the changes in the body-build from one extreme of variation to the other, as from the extreme asthenic to the extreme pyknic type.

2. It should be dimensionally homogeneous and not attempt to add horses to apples.

3. It should be capable of straightforward and simple interpretation or in short its numerical values should have a rational, as distinguished from rationalized meaning.

4. The frequency distribution of its values in large homogeneous groups should not deviate widely from the normal curve.

5. It should be based on dimensions capable of relatively easy and accurate measurement.

6. It should be simple and easy to compute (Pearl, *loc. cit.*).

Bearing in mind these desiderata and being impressed by Franzen's idea of a nutritional status along with Karl Pearson's three dimensional plea, the author

thought of utilizing measurements of the antero-posterior length of foot, the pelvic-width and the stature in a construction of a somatometric index of the body-build. The selection of these three measurements was done after having experimented with half a dozen other body-measurements and was influenced by the following argument:—

FOOT-LENGTH, PELVIC-WIDTH AND STATURE IN AN ERECT HUMAN BODY.

The assumption of a habitual erect posture by a man has resulted in an alteration in the position of internal organs and the character of the osteo-muscular support of the abdomen. It has also released his fore-limbs to take a prominent part in the evolution and development of activities which have created a wide gap between the activities characteristic of man and those of other mammals. With the carriage of the head on the spine and the maintenance of the long axis of the lower limbs in line with the long axis of the trunk, the weight of the body in the standing position is borne exclusively by the two feet. Howell (1939) describes that 'pelvic stresses are not at a right angle to the vertebral column but in a line with it'. As a result of the height becoming the longest dimension of the body, many structural alterations have followed more marked in the region of the pelvis and the feet. The pelvis is widened out in a lateral direction and the foot is shortened in an antero-posterior direction. These tendencies are seen clearly from the table* in the treatise by Humphrey (1858) and the descriptions by Sonntag (1924). These alterations were necessary for the maintenance of the erect posture and for the convenience of the plantigrade progression.

The erect human body could be compared to an erect column standing on one of its two ends. The maintenance of this posture, as Haycraft (1900) has mentioned, would only be possible if the centre of gravity of an erect column plumbs within the area of its base which would coincide with the area covered by the feet together with the area between them (foot-area or the area of under-propping). The centre of gravity in an erect human body would be situated between the pubes and buttocks, that means in the pelvis, as demonstrated by the experiments of Borellus (1679) and the brothers Weber and Weber (1836). These results were verified later by Harless (1860) and Meyer (1873).

An erect human body is always in a state of unstable equilibrium as its centre of gravity is placed above the point of its support. So any push from behind during this posture will make the body fall over the toes if it is sufficiently strong to overcome the force of gravity acting downwards through the distance between the front and back of the foot-area (Haycraft, *loc. cit.*). This distance does not exactly correspond to the antero-posterior length of the foot, as the long axis of the foot does not ordinarily lie in a sagittal plane while standing at ease but

* Foot-length in proportion to height.

(A Treatise on the Human Skeleton' by Humphrey.)

			Per cent.
Orang	25.00
Chimpanzee	21.00
Gorilla	20.59
Negro	17.90
European	16.03

makes a small angle with it. As, however, the length of the foot influences this distance, the amount of force necessary to topple the body forward must be proportional to the length of the foot. Correspondingly, any push sideways is resisted by increasing the space between the feet.

It follows, therefore, that as the pelvis is the seat of the centre of gravity, and the length of foot capable of influencing the base, both these body-parts along with the height may be included to form the basis of a study concerning the shape of the body or concerning efficiency of the human body as a mechanical machine, or concerning the distribution of soft tissues or bulk around the skeleton.

Further, it can be seen that the height of the body is at right angles to both the length of the foot and the width of the pelvis. The foot-length is at right angles to both the height and the pelvic-width; and the pelvic-width is at right angles to both the height and the foot-length. Thus height, length of the foot and pelvic-width have three dimensional dispositions in an erect human body.

CONTENTS OF THIS PAPER.

Thus assuming foot-length, pelvic-width and stature as three dimensions of an erect human body, the body-weight was studied in relation to the product of these three measurements. With some further modifications a new somatometric index was evolved.

This paper mainly deals with the results and conclusion arrived at by the author regarding the working of this new somatometric index.

MATERIAL AND METHODS.

Material.—The data used in this study are drawn from about 3,200 measurements made on boys in the primary schools of the Bombay suburban district, who were measured repeatedly at yearly intervals for three consecutive years. A further series of measurements made on 1,790 boys from two high schools in the city of Bombay along with measurements made on 300 male candidates who had presented themselves for service in a local police force, further measurements made on more than 300 college boys and 150 other individuals are included in this study. All these individuals were natural inhabitants of Western India and were ethnologically akin to each other.

Indians are not usually accustomed to the habitual wear of constricting foot-gear, in the shape of boots or shoes, like the European or American populations. The use of a foot-wear in most cases is restricted for outdoor purposes, and that too by the people who can afford to possess them. Economically poorer classes may go without any protection to the feet at all ages during all seasons. The foot-gear used by Indians which is known as *chappal* mostly consists of a leather-sole piece with one or two leather straps for instep, and another strap between the great toe and second toe.

Method.—Height, length of the foot, ilio-cristal diameter and weight of each individual were recorded. Standard instruments were used for all these measurements. The measurements were made by the author with the help of his assistants following the methods advocated by Hrdlicka (1920) and Martin (1928, quoted by Davenport, 1932-33) (Plate VI, figs. 1 and 2, and Plate VII, fig. 3).

PLATE VI.



Fig. 1.—Height is being taken with an anthropometer on the platform. The length of the left foot is being recorded in the same position.

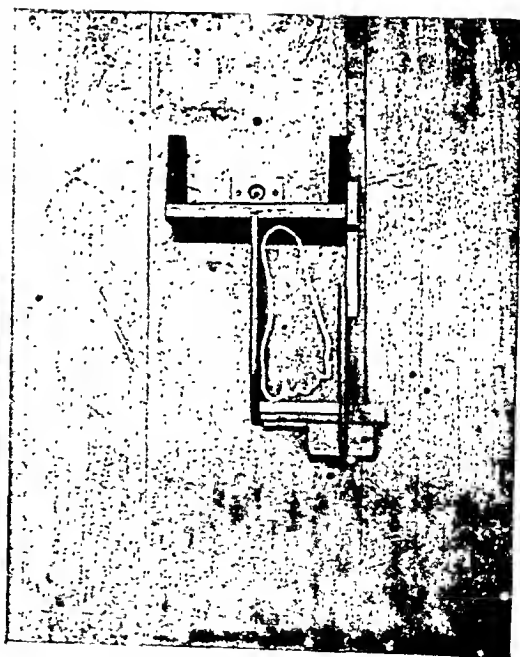


Fig. 2.—Pedometer, on the platform for recording height, in the process of foot-length being taken as the person stands with his left foot in position as shown in this picture.



Fig. 3.—Pelvic-width (ilio-cristal diameter) is being taken.



Fig. 4.—Thickness of skin and subcutaneous tissue at the right anterior axillary fold is being registered by means of craniometer.

Height and foot-length.—For these two measurements the subject was always standing on both feet which were bare on a levelled platform. Foot-length was measured according to the method suggested by Martin (*loc. cit.*) by a modified osteometric board incorporated in the platform. This enables an accurate measurement of a straight line distance between the distal tip of the first or second toe and the hindmost point of the heel of the foot in a standing posture bearing the weight of the body (Plate VI, figs. 1 and 2).

Ilio-cristal diameter.—The ilio-cristal diameter was taken by keeping two tips of the craniometer or pelvimeter on the iliac crests of both sides and moving the tips of the instruments along them to decide and record the greatest distance between them. Ilio-cristal diameter indicates the width of the pelvis (Plate VII, fig. 3).

Weight.—For the purpose of recording weights the subjects in all cases removed their clothing keeping only the underwear. The average weight of the underwear of an individual which was retained was found to be 5 oz. to 10 oz. varying according to the age of the subject, and this was determined by weighing a few samples of the underwear.

In this study the individuals ranged between the age of 5 years and over 25 years.

THE BODY-WEIGHT IN RELATION TO THE ERECT HUMAN BODY WHOSE
STATURE, PELVIC-WIDTH AND FOOT-LENGTH ARE ASSUMED
TO BE ITS THREE DIMENSIONS.

Body-weights were taken in grammes and linear measurements in centimetres.

Section I.

THE C INDEX.

A. The formula of C index.—During the early period of these investigations it was found that weight as related to the product of stature and foot-length, in the following manner, always equalled one-half of the measurement of ilio-cristal diameter in cm.

$$\frac{\text{Weight in grammes}}{\text{Stature in cm.} \times \text{Foot-length in cm.}}$$

This phenomenon led to the suggestion that if one-half of ilio-cristal diameter in cm. representing the pelvic-width was incorporated in the formula the results will be near about 1.

Relationship of the body-weight to stature, pelvic-width and foot-length is thus demonstrated by the formula:—

$$\frac{\text{Weight in grammes}}{\text{Stature in cm.} \times \frac{1}{2} \text{ Pelvic-width in cm.} \times \text{Foot-length in cm.}}$$

According to this formula an index was worked out for each individual. The lowest value of this index in this series is 0.70 and the highest is about 1.54. The mid-point of this range lies at about 1. This formula can also be expressed in the following manner:—

$$\frac{2 \times \text{Weight in grammes}}{\text{Stature in cm.} \times \text{Foot-length in cm.} \times \text{Pelvic-width in cm.}}$$

TABLE

Distribution of individuals in age

Mid-points of age groups in years.	0.70 to 0.74	0.75 to 0.79	0.80 to 0.84	0.85 to 0.89	0.90 to 0.94	0.95 to 0.99	1.00 to 1.04	1.05 to 1.09	1.10 to 1.14
4	1	...	1
5	...	2	4	2	9	15	16	8	13
6	1	...	9	9	27	57	62	49	34
7	1	8	13	41	81	104	110	68	47
8	5	10	26	91	131	155	99	69	32
9	7	13	49	104	168	153	97	52	26
10	6	28	55	112	155	133	65	33	10
11	10	34	78	128	126	81	37	16	11
12	9	23	79	110	111	58	19	16	3
13	8	24	70	83	77	48	22	10	6
14	4	25	58	74	51	31	12	4	3
15	6	24	41	42	30	22	7	6	4
16	5	8	25	37	28	19	8	5	5
17	...	7	20	23	17	19	8	2	...
18	3	1	13	19	25	21	21	6	4
19	...	2	18	20	27	27	12	8	5
20	...	1	7	31	33	34	26	15	8
21	...	2	5	18	33	27	22	11	15
22	...	1	10	17	11	31	14	18	15
23	...	1	4	9	19	13	11	9	5
24	8	9	11	8	6	11	8
25	3	7	10	8	10	7	4
A. 25	...	2	...	4	10	14	11	14	14
TOTAL ...	65	216	595	990	1,190	1,080	696	437	273

I.

groups according to the C index.

1.15 to 1.19	1.20 to 1.24	1.25 to 1.29	1.30 to 1.34	1.35 to 1.39	1.40 to 1.44	1.45 to 1.49	1.50 to 1.54	Total number of individuals in age groups.
...	2
3	2	5	79
16	10	8	3	4	289
11	11	2	1	3	501
14	8	4	1	645
9	7	1	686
4	5	1	1	1	2	611
1	1	1	524
3	1	...	2	...	1	435
...	1	...	1	350
1	263
2	...	1	1	...	1	187
1	141
1	97
2	1	1	117
1	2	1	2	1	126
3	2	...	1	1	...	162
7	3	3	1	147
4	5	...	4	1	1	132
1	4	1	2	...	1	...	1	81
1	6	68
...	3	...	2	55
5	11	2	3	90
90	83	29	25	10	8	1	1	5,789

A Myotrophic Index.

This index is referred to in the text as C index.

B. Material and methods.—The C index has been worked out in 5,789 male individuals of different ages of this series. Grouping has been done in 12 monthly age groups, with mid-points at the months of a complete year. All the persons of ages above 306 months are put in one last group. (A. 25 group in Table I).

C. Results.—Distribution of 5,789 persons in age groups is given in Table I.

Means, standard deviations, coefficients of variation and their standard errors were worked for each age group and these are reproduced in Table II :—

TABLE II.

Means, standard errors, standard deviations and coefficients of variation of C index of different age groups.

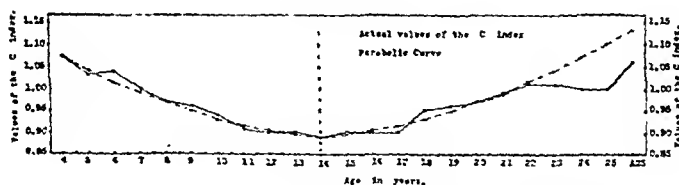
Mid-points of age groups in years.	Numbers.	Mean.	Standard error.	Standard deviation.	Coefficient of variation.
4	2	1.07
5	79	1.03	± 0.01	0.11	10
6	289	1.04	± 0.006	0.11	10
7	501	1.00	± 0.004	0.10	10
8	645	0.97	± 0.003	0.09	9
9	683	0.96	± 0.003	0.09	9
10	611	0.94	± 0.005	0.12	13
11	524	0.91	± 0.004	0.09	10
12	435	0.90	± 0.005	0.09	10
13	359	0.90	± 0.004	0.08	9
14	263	0.89	± 0.004	0.08	9
15	187	0.90	± 0.006	0.10	11
16	141	0.90	± 0.008	0.09	10
17	97	0.90	± 0.008	0.08	9
18	117	0.95	± 0.009	0.09	9
19	126	0.96	± 0.009	0.11	11
20	162	0.97	± 0.008	0.10	10
21	147	0.99	± 0.008	0.10	10

TABLE II—concl'd.

Mid-points of age groups in years.	Numbers.	Mean.	Standard error.	Standard deviation.	Coefficient of variation.
22	132	1.01	± 0.01	0.12	11
23	81	1.01	± 0.01	0.15	14
24	68	1.00	± 0.01	0.12	12
25	55	1.00	± 0.016	0.12	12
A. 25	90	1.06	± 0.012	0.12	11

It can be seen from Table II that C index at the age of four is 1.07. The number of individuals in this age group is only two, and the mean C index of this age group is not representative of this group owing to a very small number of individuals in it. From the 4th to the 7th year there is a drop of 0.07 in the mean C index and at the 8th year there is a further drop of 0.03. In the 8th and 9th year there is a decrease in the index at the rate of 0.01 every year. In the 9th and 10th year a drop of 0.02, and in the 10th and 11th a drop of 0.03 can be seen. At the 12th year it further decreases by 0.01. Between 12th and the 17th year the index is nearly constant. The 14th year is characterized by a lowest level at 0.89. In the 17th and 18th year there is a sudden rise in the index by 0.05, and thereafter roughly every year by 0.01 or 0.02 the index rises to reach a level of 1.01 at the age of 22 years. Between 22nd and 25th year again the index is nearly constant at about 1.00. In the last age group which consists of the persons above 25 years the index is, however, 1.06. These means of the different age groups are plotted in Graph 1:—

GRAPH 1.



Actual values of the C index in continuous line, and the parabolic curve of the formula $Y = 0.45 \cdot X^2$ in broken line. Parabolic curve is drawn taking the age of 14 years as 0 on the abscissa. Y represents the values of the C index and X the age in years. Actual values of the C index up to the age of 22 are distributed closely around the line of the parabolic curve, and after 22 years these are falling below the level of the parabolic curve, remaining at the same level up to the age of 25 years. In the last age group, however, the actual value has suddenly risen to 1.06. The lowest actual value of 0.89 has coincided with the lowest level of the parabolic curve at the age of 14 years.

The curve produced by plotting the means in Graph 1 looks like a hanging chain. The lowest point on the curve is at the age of 14 years. From the point of the 14th year the curve gradually rises up on both sides of that year. From the 14th year back to the 4th year on one side and from the 14th year up to the 22nd year on the other side the curve looks to be of uniform curvature. After the 22nd year, however, the curve flattens out. The extent of the curve up to the 22nd year can be divided into two symmetrical halves if an ordinate is drawn at the point of the 14th year (Graph 1). Table III shows the paired years on both sides of that year, together with the means of the C index for the respective years.

TABLE III.

Distribution of C indices around the 14th year in pairs.

Age in years.	C index.	14th year C index.	C index	Age in years.
13	0.90	0.89	0.90	15
12	0.90	...	0.90	16
11	0.91	...	0.90	17
10	0.94	...	0.95	18
9	0.96	...	0.96	19
8	0.97	...	0.97	20
7	1.00	...	0.99	21
6	1.04	...	1.01	22
5	1.03	...	1.01	23
4	1.07	...	1.00	24
...	1.00	25
...	1.06	A. 25

Mean C indices of the following pairs are equal: 13 and 15, 12 and 16, 9 and 19, 8 and 20 years. The means of the pairs of 7 and 21, 10 and 18, 11 and 17 years have a very small difference of 0.01 only and may be considered as nearly equal. The means of 6 and 22 years have a difference of 0.03. While the means of 6, 5 and 4 years are disposed along the parabolic curve, the means of 23, 24, 25 and above 25 years progressively lie below the line of the parabolic curve.

The disposition of the curve suggests that it may probably belong to some class of a parabola. The equation of a parabola is $Y = ax^n$, where 'a' and 'n' are constants, and 'x' the values along the abscissa and 'Y' the values along the ordinate. The age in years is to be plotted along xx' and yy' is to be made to stand at the point of 14 years on which the numerical values of the index are to be plotted. Taking two or three different values for 'x' and 'y', the values for the constants were worked out and determined. The average value for 'n' was found to be about 1.6, and the value for 'a' was found to be about 0.0045. The equation for this parabolic curve thus is: $Y = 0.0045 \times X^{1.6}$, which means that C index varies directly according to the age distance from the 14th year.

Using this formula, the calculated values for 'Y' were determined for all the age groups and are reproduced in Table IV along with the actual means for the respective age groups:—

TABLE IV.

Actual and calculated values of the C index at different ages.

Mid-points of age groups in years.	Calculated values ($0.89 + Y$).	Actual values of this study.
4	1.07	1.07
5	1.04	1.03
6	1.015	1.04
7	0.99	1.00
8	0.97	0.97
9	0.95	0.96
10	0.931	0.94
11	0.915	0.91
12	0.904	0.90
13	0.89	0.90
14	0.89	0.89
15	0.89	0.90
16	0.904	0.90
17	0.915	0.90
18	0.931	0.95
19	0.95	0.96
20	0.97	0.97
21	0.99	0.99
22	1.015	1.01
23	1.04	1.01
24	1.07	1.00
25	1.10	1.00
A. 25	...	1.06

In Graph 1 values of C index according to the equation $0.89 + Y$ in which $Y = 0.0045 \times X^{1.6}$ are plotted for different age groups resulting in a curve and actual mean C indices have been superposed over the curve (Table IV). The C indices of 4, 8, 11, 12, 14, 16, 20, 21 and 22 years are almost falling on the curve, the C indices of 7, 9, 10, 13, 15, 18 and 19 years are also nearly falling on the curve though they are above the curve by 0.01 only. The C index of 6 years only is in excess of the curve by 0.025. The C indices of 5 and 17 years are falling below the curve by 0.01 only. The C indices of 23, 24, 25 years and the last age group are falling short of the curve by 0.03 or more.

It can be deduced, therefore, that C indices of persons of 22 years and below closely fit on a parabolic curve whose equation is $Y = 0.0045 \times X^{1.6}$, where 'X' is a distance in years from the 14th year, and 'Y' is the increase in 'C' index on the basic index 0.89 at the 14th year.

D. Comments.—From the results, the following observations may be arrived at:—

(i) Distribution of C index is between 0.70 and 1.54 (Table I), probably revolving around 1.

(ii) Means of C indices of different age groups, if plotted, lie in a parabolic curve, the lowest point on the parabola lying at the 14th year.

(i) If the observation that the values of C index tend to revolve around 1 is true, then certain further deductions become possible. In this study, height, foot-length and pelvic-width have been adopted as workable dimensions of an erect human body. If the erect human body would be a perfect cylinder with the above lengths as its dimensions, and if the specific gravity of the human body would be 1 (actually it is slightly more than 1, if the volume of the residual air in the lungs is deducted from the volume of the body), then the product of these three dimensions in cm. would be equal to weight of the human body in grammes. The conditions of the human body are, however, otherwise. A human body is not a regular geometrical pattern of three specific dimensions. Hence assumption of these body diameters as three dimensions probably is half correct. A formula of a volume or weight of a cylinder is volume or weight (if specific gravity is 1) = length \times depth \times width. The formula of C index in comparable terms is V or W (if specific gravity is 1) = $\frac{1}{2}$ ($L \times D \times W$), or in other words this formula could be written as follows:—

$$0.7938 \text{ of } L \times 0.7938 \text{ of } D \times 0.7938 \text{ of } W = V \text{ or } W \text{ (if specific gravity is 1).}$$

The three body measurements, namely height, pelvic-width and foot-length, seem to be actually in excess by nearly their one-fourths of the hypothetical actual dimensions.

The human cylinder is made up of skeleton and soft tissues and hence any decrease or increase in weight is primarily due to the wastage or increase of soft tissues, while the assumed three dimensional measurements remain constant.

(ii) The tendency of C indices to lie in a parabolic curve is an interesting phenomenon and this parabolic tendency is observed prior to 22 years.

The normal healthy individual undergoes changes in the body-bulk from infancy to adult age. A healthy infant is plump. At the age of adolescence, which is near about 14 years, an individual becomes thin. As the adult age is reached a person normally puts on flesh. Initial plumpness, intermediate thinness at the adolescence and putting on flesh in adult age, seem to be synchronizing with the parabolic curve of the C index.

Section II.

C INDEX IN NORMAL INDIVIDUALS.

An attempt is made in this Section to see whether the lower values of the C index can be attributed to the smaller body-bulk and higher to the larger body-bulk.

For this purpose some 1,123 individuals of this study were observed with the idea of determining roughly the normal adequacy of weight of each individual. These observations were made on each individual independently by two medical observers (G. M. K. and G. N. W.).

The procedure of taking observations consisted of mental estimations. The criteria for estimations were determined beforehand and consisted of determining the degree of body-bulk. While determining the bulk, due regard was given to the condition of health and to the presence of any constitutional derangement. The mental impression created by these observations as to whether the individual can be considered possessing adequate weight indicating a good nutritional status or not was recorded by means of the following four notations :—

1. Poor (P)
2. Barely normal (BN)
3. Normal (N)
4. Above normal (AN)

The remark normal indicates that the body-weight was adequate and the condition of health was satisfactory. Barely normal indicates that the body-weight and the health could be said neither poor nor normal. Poor indicates a decidedly inadequate weight associated with poor condition of health and above normal indicates obesity.

There was an agreement of remarks between the two observers on 680 persons, and a disagreement on 433. The disagreement was mostly of a difference of one grade only (Table V) :—

TABLE V.

First observer.	Second observer.	
Poor	Poor ...	195 (P-P).
„	Barely normal ...	142 (P-BN).
Barely normal	„ „ ...	140 (BN-BN).
„ „	Normal ...	244 (BN-N).
Normal	„ ...	292 (N-N).
„	Above normal ...	47 (N-AN).
Above normal	„ „ ...	63 (AN-AN).
	TOTAL ...	1,123

* Abbreviations used in the text.

Distribution of persons according to the remarks of mental estimations and ages is given in Table VI:—

TABLE VI.

Distribution according to ages and mental estimations.

Mid-points of age groups in years.	P-P	P-BN	BN-BN	BN-N	N-N	N-AN	AN-AN	Total.
5	...	1	3	...	3	7
6	4	2	4	6	11	...	2	29
7	12	7	10	9	14	2	2	56
8	16	14	8	15	8	61
9	13	5	8	18	17	2	...	63
10	16	14	5	10	13	2	1	61
11	22	11	11	11	21	2	...	78
12	16	11	10	16	12	1	1	67
13	28	15	19	20	12	6	7	107
14	16	15	13	24	24	3	3	98
15	10	14	10	21	19	6	9	89
16	8	9	8	21	25	6	5	82
17	6	6	4	10	14	2	2	44
18	1	3	3	6	11	4	3	31
19	3	2	1	7	6	1	2	22
20	7	1	2	5	11	1	1	28
21	2	5	5	9	11	1	3	36
22	6	1	2	11	27	2	3	52
23	1	2	6	7	5	...	3	24
24	1	6	10	...	5	22
25	1	2	3	6	7	3	1	23
A. 25	6	2	5	6	11	3	10	43
TOTAL ...	195	142	140	244	292	47	63	1,123

It may be admitted that the number of individuals in each individual class has been reduced as the number of 1.123 has been distributed in 22 groups and 7 classes.

TABLE VII.

Means of the C index of age groups related to mental notations.

Mid-points of age groups in years.	P-P	P-BN	BN-BN	N-BN	N-N	AN-N	AN-AN
5	...	1.02	0.97	...	1.00
6	0.91	1.00	0.98	1.00	1.04	...	1.02
7	0.91	0.94	0.96	0.98	1.00	1.02	1.05
8	0.90	0.90	0.98	0.93	0.96
9	0.91	0.91	0.90	0.92	0.93	1.04	...
10	0.85	0.88	0.90	0.94	0.97	1.00	1.32
11	0.86	0.85	0.86	0.90	0.98	1.02	...
12	0.87	0.88	0.90	0.88	0.94	0.87	0.97
13	0.84	0.88	0.90	0.90	0.93	0.98	1.07
14	0.84	0.88	0.88	0.88	0.94	0.90	1.07
15	0.77	0.83	0.90	0.88	0.94	0.93	1.14
16	0.83	0.86	0.86	0.89	0.93	0.93	1.15
17	0.82	0.82	0.83	0.93	0.96	0.90	1.07
18	0.72	0.75	0.84	0.89	0.96	1.09	1.16
19	0.90	0.87	0.82	0.91	1.00	0.97	1.15
20	0.89	0.87	0.92	0.89	1.00	0.87	1.47
21	0.82	0.91	0.97	0.93	1.08	1.02	1.26
22	0.85	0.87	0.92	0.94	1.07	1.27	1.22
23	0.92	0.82	0.90	0.90	1.10	...	1.26
24	0.92	0.95	1.07	...	1.22
25	0.92	0.82	0.96	0.95	1.04	1.14	1.22
A. 25	0.97	0.92	0.94	0.93	1.07	1.15	1.18

From Table VII it will be seen that generally the numerical values of the C index go on increasing according to the classes in the order: 1. P-P, 2. P-BN, 3. BN-BN, 4. BN-N, 5. N-N, 6. N-AN, 7. AN-AN.

Out of 7 classes the 4 classes in which agreement has been reached between two observers may be considered in some details.

Poor-Poor.—There are 195 individuals in this class. 7th to 15th year age groups of this class have more than 10 individuals in each. 16th year age group has 8. The remaining age groups have very few individuals. The C indices in 7th to 15th year age groups are 0.91, 0.90, 0.91, 0.85, 0.86, 0.87, 0.84, 0.84, 0.77, respectively. That of the 16th year is 0.83. The 15th year shows the lowest index, which is 0.77. The decline from 0.91 to 0.77 observed in the 7th to 15th year age groups compares favourably with the similar decline in same age groups observed in the general study of the C index in Section I of this part. The numerical values for the C index of the P-P class are all decidedly lower than the averages of the general group (Table II). The lowest numerical value for the C index in the general study of the C index is at the 14th year. In P-P class it is at the 15th year.

Barely Normal-Barely Normal.—This group has 140 individuals. 7, 11, 12, 13, 14 and 15 years age groups have 10 or more individuals. The values of the C index in this class seem mostly more than those of P-P class and less than those in the N-N class.

Normal-Normal.—There are 292 individuals in this class. All the age groups except 5, 8, 19, 23 and 25 years age groups have more than 10 individuals in each. The C index for the 6th year in this class is 1.04, which decreases to 1.00 at the 7th year. The values of the C index in the later years are as follows: In the 9th year 0.93, in the 10th year 0.97, in the 11th year 0.98, in the 12th year 0.94, in the 13th year 0.93, in the 14th and the 15th year 0.94, in the 16th year 0.93, in the 17th and the 18th year 0.96, in the 20th year 1.00, in the 21st year 1.08, in the 22nd year 1.70, in the 24th year and the last age group 1.07. The averages in this class are mostly more than the averages of BN-BN class. The lowest numerical value in this class is 0.93 and is at the 13th and 15th years.

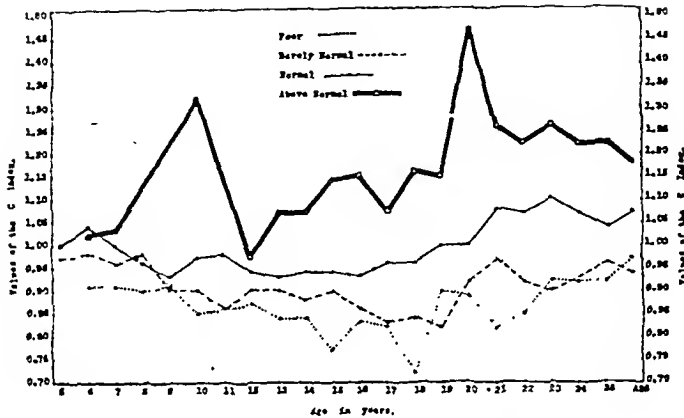
Above Normal-Above Normal.—All the age groups except the last one in this class consist of less than 10 individuals. The average for the last age group is 1.18. The averages in this class are all of them except that of the 6th year more than those of all other classes.

The means of all the four classes have been plotted in Graph 2.

It will be seen from Graph 2 that the curve of P-P class is decidedly always lower than the curve of N-N in all age groups. The curve of the BN-BN class is higher than the curve of the P-P class and lower than the N-N class. The curve of the N-N class is higher than both the P-P and BN-BN classes. While the curve of the BN class has touched or even gone below the curve of P class at 9, 11, 19 and 23 years and the years

above 25 it has only once, at the 8th year, crossed the N curve and is above it.

GRAPH 2.



Values of the C index according to the notations regarding the constitution (and of those cases where agreement has been reached between two medical observers) from 5 to 25 years and above 25 years. Values of the C index of the P class in dotted line, of the BN in broken line, of the N class in a continuous line and of the AN class indicating obesity in a thick line. Note the values of the P class are lowest at all ages than the N class. Values of the BN class are between the N and the P class excepting at the age periods of 8, 9, 11, 19, 23 and 25. Only once at 8 years the value of the BN class has exceeded the value of a N class of that age, and at 9, 11, 19, 23 and 25 years slightly lower than the values of the P class of the respective age periods. Values of the C index for the N class are always above the P, and except at 8 years always above the BN class and always below the values of the C index for the AN class except at 6 years. Values of the AN class are always above all classes except at the 6th year.

All the curves seem to have slight curving tendencies and a terminal flattening after the 22nd year.

DISCUSSION.

In the P-P class the values for the C index are the lowest, in the BN-BN class they are a little higher, in the N-N class they are still higher and in the AN-AN class highest. It can, therefore, be considered that the numerical values of the C index march in an unidirectional manner from one extreme of variation to the other, as from the P to the AN class.

The values of N class seem to be in advance of P class on an average by 0.10.

THE COMPARISON OF NORMAL-NORMAL AVERAGES WITH THE AVERAGES
IN THE GENERAL STUDY (TABLES II AND VII).

The level of the C index in general group between 11 and 17 years has remained nearly stationary at the level of 0.90. It has dropped to 0.89 at the 14th year. In N-N class also, the C index can be seen to remain stationary between 12 and 16 years, and at the level of 0.94. It has dropped to 0.93 level at 13th and 16th year. Between 11 and 17 years in general study (Table II) and between 12th and 16th year in the N-N class also (Table VII) the values of the C index are at their lowest. It can, therefore, be said that the relative body-bulk seems to be at its lowest between 12 and 16 years during life. The difference between the lowest value in the general study at the 14th year and that in the N-N class at 13th or 16th year is 0.04. The average of the differences of the C indices in the general study and N-N class for all age groups is 0.034, the N group being nearly always more than the general group. Only at the ages of 5, 6, 7, 8, 9 years one observes that the averages of the general group are slightly in excess or equal to the averages in the N-N class.

It may, therefore, be justifiable to assume that the numerical values of C index of individuals with normal body-bulk (normal character determined by two observers) are in excess by 0.034 of the values of C indices of individuals in the general group (Table II). For actual practice, however, the figure 0.04 may be adopted in place of 0.034.

It will have been observed that by plotting the different constitutional groups (Graph 2) irregular patterns are produced, showing slight tendencies towards parabolic curving. The curves could not be well marked probably because of limited number of individuals in these groups. Judging, therefore, by the results of general study (Graph 1) it may be assumed that age differences in C index in individuals of different body-bulks such as P, N, etc., ought to manifest also in parabolic curves.

To arrive at a theoretical C index of normal body-bulk in different age groups younger than 22 years (here the parabolic formula is applicable), one has to calculate an index according to the formula, 0.89 *plus* ($Y = 0.0045 \times X^{1.6}$) and add to the result 0.04.

C indices of age groups older than 22 years are not disposed parabolically (Graph 1) and also are not in a straight line. This irregularity may be due to less number of individuals in those groups. So in individuals of 23, 24, 25 years the value of C index for 22 years may be adopted.

In case of persons above 25 the C index for normal body-bulk should be 1.10, being in excess by 0.04 of the average 1.06 in that group of the general study.

In Table VIII the following items are given side by side:—

1. Actual numerical values of C index for normal body-bulk judged by two observers.

2. Theoretical numerical values of C index for normal body-bulk calculated according to the formula of the parabolic curve of general study (Graph 1) and

adding 0.04 to the results for age groups up to 22 years. For 23, 24 and 25 years, the value for 22 years being retained. For the age group above 25 years, by adding 0.04 to the value of the same group in the general study.

3. Numerical values of C index of general study (Table II).

TABLE VIII.

Mid-points of age groups in years.	C index normal, actuals (N-N—Table VII).	C index normal, theoretical.	C index general group (Table II).
4	...	1.11	1.07
5	1.00	1.07	1.03
6	1.04	1.05	1.04
7	1.00	1.03	1.00
8	0.96	1.01	0.97
9	0.93	0.99	0.96
10	0.97	0.97	0.94
11	0.98	0.95	0.91
12	0.94	0.94	0.90
13	0.93	0.93	0.90
14	0.94	0.93	0.89
15	0.94	0.93	0.90
16	0.93	0.94	0.90
17	0.96	0.95	0.90
18	0.96	0.97	0.95
19	1.00	0.99	0.96
20	1.00	1.01	0.97
21	1.08	1.03	1.01
22	1.07	1.05	1.01
23	1.10	1.05	1.01
24	1.07	1.05	1.00
25	1.04	1.05	1.00
A. 25	1.07	1.10	1.06

Age corrections in C index.

As values of C index for different age groups have a tendency to lie in a parabolic curve, comparison of body-bulk as indicated by C index of different ages could only be possible if age corrections were to be incorporated in C index.

Age corrections would be height differences of points at different ages on the arms of parabola, from the lowest point on the curve at the 14th year, in terms of numerical values of C index.

From the theoretical values for normal body-bulk (Table VIII, middle column) the following age corrections are thus arrived at:—

TABLE IX.
Age corrections for C index.

Age in years.	Age in years.	Age in years.
5 ... 0.14	12 ... 0.01	19 ... 0.06
6 ... 0.12	13 ... 0.00	20 ... 0.08
7 ... 0.10	14 ... 0.00	21 ... 0.10
8 ... 0.08	15 ... 0.00	22 ... 0.12
9 ... 0.06	16 ... 0.01	23 ... 0.12
10 ... 0.04	17 ... 0.02	24 ... 0.12
11 ... 0.02	18 ... 0.04	25 ... 0.12
...	A. 25 ... 0.17

Section III.**MYOTROPHIC INDEX.**

C index is an indicator of total body-bulk as related to skeletal dimensions (height, foot-length and pelvic-width). It would not naturally discriminate between the body-bulk made up of excessive fat and that made up of scanty fat and more of the other tissues.

Two individuals may have the same body-weight, and still in one there might be more of fat and in the other fat may be scanty.

According to Franzen's idea, if quantity of fat and muscle could be measured directly and related to the skeleton, an estimate of nutrition could be possible.

No known definite anthropometric measure, which would give a fair indication of the state of voluntary muscles in the body, is available at present. This type of an estimation does not seem possible because in the first instance not one muscle in the body is approachable for its fairly accurate measurement in any one of

the dimensions as it is deep to the skin and subcutaneous tissue, and secondly a measurement of one dimension of any one muscle in the body would not serve as an index of the condition of the whole muscular system in the body.

An indicative measure of adiposity is, however, possible. A measure of adiposity may indicate as to whether adipose tissue in the body is scanty or is in excessive quantity. If, therefore, it is found by some means that the adipose tissue in the body is scanty and at the same time the numerical value of C index of the individual is not of poor class, then, in absence of any other pathological conditions, the body-bulk which this index shows as normal is likely to be made up by more of the muscular tissue, assuming the skeletal weight and weights of organs being constants.

Adipose tissue of fat gets deposited in the body at various places. It accumulates in and around organs, and is deposited under the skin. The deposit of fat under the skin is not of uniform thickness in all places. At some places even in non-obese persons, fat can be seen normally deposited, while at other places it is not normally found. In conditions of obesity the fat gets deposited all round under the skin, and would naturally increase in amount where it is normally present and at places where it is not present it will make its appearance in varying thickness. Therefore, if one constant point is selected where the thickness of the fat could be measured in all bodies, the variation of thickness at that spot would be a fair indication of adiposity in that body. Such a spot should be easy to handle. Mid-point of the anterior axillary fold of the right side is, in the author's opinion, the most convenient spot where an indicative thickness of adipose tissue in a body can be measured. The fold can be gripped in the fingers of the left hand of the measurer, while the right arm is abducted to nearly a right angle. The grip on the fold should be firm, and with firmness the fold should be sufficiently pulled down till the underlying pectoralis major muscle slips out of the fold. The fold thus devoid of a muscle should be measured in thickness by a pair of calipers (Plate VII, fig. 4, see opposite p. 299). Measurement of thickness of skin and subcutaneous tissue at different spots on the body has been attempted by Kornfeld (quoted by Bigwood, 1937) for a different purpose.

Recently, attempts have been made by Behnke, Feen and Welham (1942) to determine the obesity. The determination of obesity of a person has been proposed by them on the numerical values of the specific gravity of healthy men. These authors weighed the persons inside and outside water and have computed the specific gravity from the two readings. They have assumed that excess fat was the prime factor governing the level of the specific gravity. The hydrostatic methods for finding the specific gravity are, however, not possible to be used in cases of children and neurotic persons.

For the purposes of this study a measurement of the skin and subcutaneous tissue was made on 940 individuals of different ages.

Along with the measurement of the fat and subcutaneous tissue, the condition of obesity was attempted of persons to be mentally estimated by also feeling the anterior abdominal wall and the other easily accessible parts of the body. Thin supple feel of the skin in a pinch on the abdominal wall was considered to be 0 grade obesity. Thicker feels of the skin were put in gradually increasing

grades of 1, 2, 3, 4 and 5. While 0 grade indicates a non-obese condition, the grade 5 indicates extreme obese condition of the present series. Correlation of these remarks in grades by manual estimation of obesity, with actual measurements of the thickness of the skin and subjacent tissue at the anterior axillary fold, is shown in Table X:—

TABLE X.

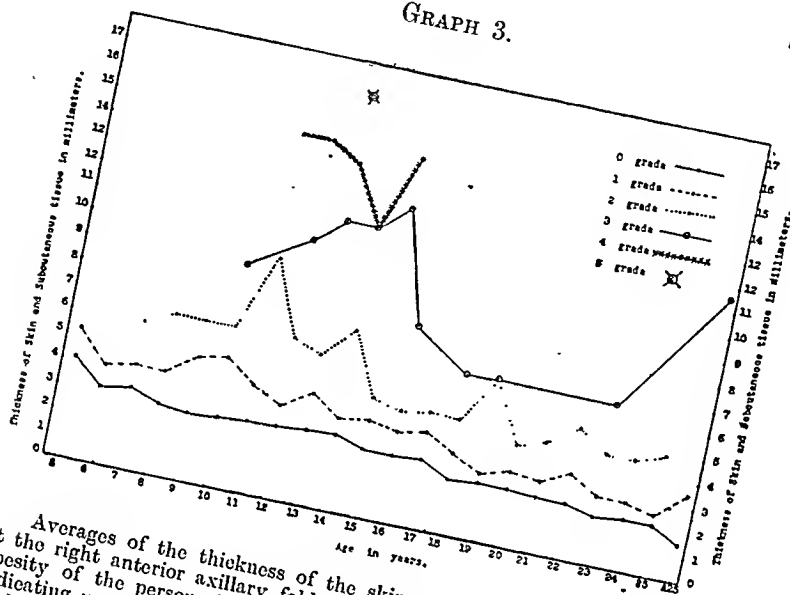
Averages of thickness of the subcutaneous tissue at the anterior axillary fold, according to the remarks about obesity.

(In brackets are given numbers of individuals.)

Mid-points of age group in years.	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
5	4.06 (9)	5.25 (4)
6	3.04 (26)	4.00 (6)
7	3.3 (34)	4.25 (8)	6.0 (2)
8	2.9 (36)	4.2 (14)	6.5 (1)
9	2.75 (30)	5.0 (14)	6.5 (5)
10	2.84 (29)	5.25 (12)	6.5 (1)	9.0 (2)
11	2.9 (28)	4.4 (13)	9.5 (1)	...	14.5 (2)	...
12	2.95 (38)	3.8 (14)	6.6 (10)	10.5 (1)	14.5 (1)	...
13	3.04 (30)	4.59 (24)	6.1 (5)	11.5 (2)	13.84 (3)	16.5 (1)
14	3.05 (29)	3.8 (29)	7.32 (11)	11.5 (2)	11.5 (2)	...
15	2.72 (27)	3.96 (26)	4.84 (3)	12.5 (1)	14.5 (1)	...
16	2.73 (17)	3.71 (19)	4.64 (7)	8.0 (7)
17	2.85 (17)	3.96 (13)	4.75 (4)
18	2.29 (23)	3.37 (23)	4.68 (11)	6.5 (1)
19	2.34 (25)	2.72 (31)	6.5 (2)	6.5 (1)
20	2.35 (20)	3.1 (30)	4.13 (8)
21	2.24 (23)	2.95 (18)	4.5 (7)
22	2.27 (17)	3.5 (12)	5.3 (5)
23	1.97 (13)	2.84 (9)	4.5 (3)	6.5 (1)
24	2.04 (13)	2.8 (10)	4.5 (4)
25	2.1 (5)	2.5 (1)	4.87 (3)
A. 25	1.5 (1)	3.5 (1)	...	11.5 (1)

The graphs drawn from these observations show that higher degrees of obesity as estimated manually are associated with greater thicknesses at the axillary fold (Graph 3):—

GRAPH 3.



Averages of the thickness of the skin and the subcutaneous tissue at the right anterior axillary fold as related to the remarks about the obesity of the persons between ages of 5 and above 25 years. 0 grade indicating non-obesity is represented in a continuous line, grade 1 in a broken line, grade 2 in a dotted line, grade 3 in a continuous line with circles, grade 4 in a starred line, and grade 5 in a double circled star. 0 line shows nearly a straight line, except that before 15 years averages are about 3 mm., the maximum being 4 mm. at the age of 5 years. After 15 years the cutaneous thickness seems to be getting less than 3 mm., reaching about 1.5 mm. in the last age group. Successive higher grades of obesity show successive higher levels of thickness of the skin and the subcutaneous tissue.

It may, therefore, be assumed that measurement of thickness at the anterior axillary fold may be adopted to indicate the condition of adiposity of the human body.

It seems that in early childhood before the 8th year of life the thickness of skin and subcutaneous tissue is slightly greater than in adults in non-obese persons. In non-obese persons of fifteen years and above the axillary cutaneous thickness does not exceed 3 mm. In persons below the age of 15 years the thickness in non-obese does not exceed 5 mm.

It is now to be seen whether as the thickness of the skin and subcutaneous tissue increases the body-bulk also increases, and if it increases in what proportion

it does so. Table XI gives the distribution of the C index according to the measurement of skin and subcutaneous tissue in these 940 persons:—

TABLE XI.

Fat measurement and the average C index of each group.

(Distribution of individuals is given in Table XII-b).

Mid-points of age groups in years.	Up to 0.5 mm.	1-2 mm.	3-4 mm.	5-6 mm.	7-8 mm.	9-10 mm.	11-12 mm.	13-14 mm.	15-16 mm.	17-18 mm.	19-20 mm.	21-22 mm.
5	...	1.10	1.02	1.12	1.17
6	1.02	0.99	1.02	1.13	1.07
7	...	0.98	0.99	1.03	1.12
8	0.98	0.94	0.94	1.02
9	1.00	0.92	0.93	0.98	1.02
10	...	0.87	0.90	0.96	0.97	1.12
11	...	0.86	0.88	0.90	...	1.09	1.12
12	...	0.86	0.88	0.92	1.02	1.12	...	1.12
13	...	0.81	0.85	0.95	1.02	1.00	0.87	...	1.02	1.07
14	...	0.83	0.85	0.92	1.04	1.00	1.04	1.12
15	...	0.82	0.88	0.88	0.77	...	1.12	1.12
16	...	0.83	0.91	0.99	0.97
17	...	0.87	0.88	0.94	0.92
18	...	0.91	0.93	0.99	0.92
19	...	0.90	0.92	0.95	1.02
20	...	0.93	0.95	1.02
21	...	0.92	0.94	0.94
22	...	0.89	0.94	0.98
23	...	0.91	1.00	1.07
24	...	0.91	0.88	1.06
25	...	0.91	0.94	1.02
A. 25	...	0.97	0.92	1.07

By giving age corrections (Table IX) to the values of *C* index in Table XI, Table XII-a is also reproduced immediately afterwards:—

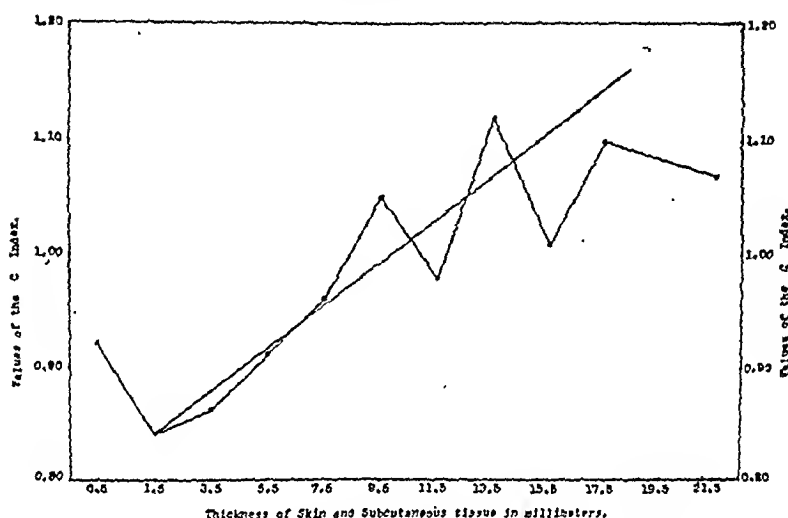
TABLE XII-a.

Fat measurement and the averages of the C index with age corrections.

by the 3 inches & under with age corrections.													
Mid-points of age groups in years.	Up to 0.5 mm.	1-2 mm.	3-4 mm.	5-6 mm.	7-8 mm.	9-10 mm.	11-12 mm.	13-14 mm.	15-16 mm.	17-18 mm.	19-20 mm.	20-22 mm.	
5	...	0.96	0.88	0.98	1.03	
6	0.90	0.87	0.90	1.01	0.95	
7	...	0.88	0.89	0.93	1.02	
8	0.90	0.86	0.86	0.94	
9	0.96	0.86	0.87	0.92	0.96	
10	...	0.83	0.86	0.92	0.93	1.08	
11	...	0.84	0.86	0.88	...	1.07	1.10	
12	...	0.85	0.87	0.91	1.01	1.11	...	1.11	
13	...	0.81	0.85	0.95	1.02	1.00	0.87	...	1.02	1.07	
14	...	0.83	0.85	0.92	1.04	1.00	1.04	1.12	
15	...	0.82	0.88	0.88	0.77	...	1.12	1.12	
16	...	0.82	0.90	0.98	0.96	
17	...	0.85	0.86	0.92	0.90	
18	...	0.87	0.89	0.95	0.88	
19	...	0.84	0.86	0.93	0.96	
20	...	0.85	0.87	0.94	
21	...	0.82	0.84	0.84	
22	...	0.77	0.82	0.86	
23	...	0.79	0.88	0.95	
24	...	0.79	0.76	0.94	
25	...	0.79	0.82	0.90	
A. 25	...	0.80	0.75	0.90	
Averages	...	0.92	0.84	0.86	0.92	0.96	1.05	0.98	1.12	1.02	1.10	...	1.07

The averages of C indices of the different groups with different cutaneous thicknesses are worked out; and are given at the bottom of Table XII-a. The age factor being eliminated by age corrections, values of C index of all age groups could directly be correlated to the thickness of fat as represented in Graph 4:—

GRAPH 4.



Averages of the values of the C index of persons with different thickness of the skin and subcutaneous tissue beginning from 0.5 mm. to 21.5 mm. The values of the C index are given as age corrections before plotting. The thickness up to 0.5 mm. is associated with 0.92 C index. From 1.5 mm. to 9.5 mm. of cutaneous thickness, C index has also risen increasingly in level. The curve is disposed mostly around the straight line superposed.

From Graph 4 it appears that a complete absence of subcutaneous fat at the anterior axillary fold is associated with relatively higher C index. At 1.5 mm. thickness C index is 0.84, and further as the cutaneous thickness increases up to 9.5 mm., C index also increases progressively up to 1.05. In later groups of 11.5 mm. and 13.3 mm. there are 5 and 3 individuals respectively, and the last two groups have 1 each. It is quite likely that the later undulation of the line may be due to the small number of individuals in those groups (Table XII-b).

A thick straight line has been superposed. Around this line, points of C indices up to 9.5 mm. thickness seem to be closely scattered, and those of 11.5 mm. and 13.5 mm. group are seen around the line but at some distance. The groups of 15.5 mm., 17.5 mm. and 21.5 mm. thickness having 1 individual in each could not be considered as representative of those groups (Table XII-b):—

TABLE XII-b.

[illegible]

Mid-points of age groups in years.	0	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22
5	...	3	6	2	2
6	1	10	16	3	1
7	...	14	24	5	1
8	4	16	24	6
9	1	18	18	9	2
10	...	19	15	8	1
11	...	21	19	3	...	1
12	...	28	23	8	2
13	...	19	27	9	1
14	...	22	32	9	3	1	1
15	...	25	25	5	1	1
16	...	17	27	2	2
17	...	14	16	2	1
18	...	33	13	10	1
19	...	37	13	2	1
20	...	32	24	2
21	...	32	12	4
22	...	21	8	5
23	...	18	6	2
24	...	20	3	4
25	...	6	2	1
A. 25	...	1	1
TOTAL	...	426	354	101	19	13	5	3	1	1	...	1

The thick line starts at 0.84 C index at 1.5 mm. thickness, and runs straight with an upward inclination to 1.07. C index at 13.5 mm. of cutaneous thickness. Thus, as the cutaneous thickness has increased from 1.5 mm. to 13.5 mm., the C index has increased from 0.84 to 1.07. Increase in cutaneous thickness is 12 mm. and increase in the C index is 0.23. For an increase of 1 mm. in cutaneous thickness there has been a rise of 0.02 of the C index.

If from the C index of an individual a figure arrived at by multiplying 0.02 by the cutaneous thickness in mm. is subtracted, the remainder of the C index would thus be representative of the bulk of the body which is constituted by the remaining tissue (devoid of fat) and muscles. This could be called as an adiposity correction.

If the argument supporting this thesis is correct, then C index of an individual, with an age correction, and a subtraction for body-fat would be indicative of body-bulk devoid of fat of which muscles form a major portion. With these modifications, C index could be called as a myotrophic index. Myotrophic index would be :—

$$\frac{2 \times \text{Weight in grammes}}{\text{Height in cm.} \times \text{Foot-length in cm.} \times \text{Pelvic-width in cm.}} - \text{Age correction} - \text{Adiposity correction.}$$

Section IV.

PRACTICAL APPLICATION OF THE MYOTROPHIC INDEX.

An account of practical application of this proposed myotrophic index and the results arrived at is given in this Section.

Measurements of 62 members from a local gymnasium were taken, almost on bare bodies. Out of the 62, 39 were daily taking exercise for more than a year, which consisted of wrestling, single bar, double bar, dunds (balancing the body on all fours on the ground and then raising and lowering it alternately), baithaks (sitting and standing alternately) and other sports. The remaining 23 were only beginners about one month. Most of the members of this gymnasium belonged to middle classes, such as teachers, clerks, etc.

Age of the members ranged from 14 to 45 years.

RESULTS.

In Tables XIII and XIV the thickness of the skin and subjacent tissue, non-modified C indices, C indices with age corrections, adiposity corrections and myotrophic indices of persons of exercising and non-exercising groups are respectively given. In Table XV comparative distribution of the thickness of the skin and subjacent tissue in exercising and non-exercising groups is given. In Table XVI comparative distribution of the C index with age corrections is given. In Table XVII the comparative distribution of the myotrophic index in exercising and non-exercising groups is given.

(a) *Exercising group.*—In this group, mean C index with age correction is 0.891 and the mean myotrophic index is 0.855. The mean thickness of the skin and

subjacent tissue at the anterior axillary fold is 1.82 mm. (Tables XIII, XV, XVI and XVII).

(b) *Non-exercising group*.—In this group, mean C index with age correction is 0.896 and the mean myotrophic index is 0.789. The mean thickness of the skin and subjacent tissue at the anterior axillary fold is 5.13 mm. (Tables XIV, XV, XVI and XVII).

	Exercising group.	Non-exercising group.
Mean C index with age correction
Myotrophic index	0.891	0.896
Mean subcutaneous thickness	0.86	0.79
	1.82 mm.	5.13 mm.

DISCUSSION.

The influence of habitual exercise is manifested in the reduction of thickness of the skin and subjacent tissue. In the exercising group, out of 39 persons 37 have thickness up to 3 mm. Only 2, one with 4 mm. and the other with 7 mm., have more than 3 mm. of subcutaneous thickness. In the non-exercising group of 23 persons there is a wide scattering of samples up to 16 mm. of thickness (see Table XV).

While the C index with age correction in two groups is nearly the same, indicating that relative body-bulk of both the groups is equal, the myotrophic index of the exercising group is greater by 0.066 than that of the non-exercising group. This indicates that, while the relative body-bulk is equal in both these groups, the muscular bulk is more in proportion in the exercising group at the cost of adipose tissue. In non-exercising group the same amount of body-bulk is made by a greater proportion of adipose tissue and less amount of muscular tissue than in the exercising group.

The difference of 0.066 between the myotrophic indices of the exercising and non-exercising groups is less by 0.034 than the average difference of 0.10 between the C indices of poor and normal groups (see page 311).

If age corrections are given, the average C index of normal persons would be 0.93 (see Tables VIII and IX). The average myotrophic index in exercising people is 0.86. The value of the myotrophic index in exercising group is lower than the normal C index and this is due to the fact that the average C index in normal persons is without any adiposity correction, while the myotrophic index is with an adiposity correction. Assuming, therefore, that in the normal group of persons

the average thickness of skin and subcutaneous tissue would be 3 mm. for want of actual measurements—this can be assumed as nearly all persons in the exercising group had not more than 3 mm. thickness. If an adiposity correction is given to the average C index of normal group, then by deducting 0.02×3 which is 0.06 from 0.93, we get 0.87 as the myotrophic index for the normal group, and this compares favourably with 0.855 average myotrophic index in the exercising group.

The myotrophic index picks out muscular persons and separates out obese and underweighted ones efficiently.

TABLE XIII.

Age, thickness of the skin and subjacent tissue, C index, C index with age correction, adiposity correction and the myotrophic index of each person who was regularly taking exercise.

Serial No.	Age in years.	Skin and subcutaneous tissue thickness in mm.	C index.	C index with age correction.	Adiposity correction.	Myotrophic index.
1	17	1	0.97	0.95	-0.02	0.93
2	18	1	1.01	0.97	-0.02	0.95
3	19	1	1.11	1.05	-0.02	1.03
4	19	2	0.91	0.85	-0.04	0.81
5	19	2	0.93	0.87	-0.04	0.83
6	19	0.5	1.04	0.98	-0.00	0.98
7	19	1	1.03	0.97	-0.02	0.95
8	20	1	0.90	0.82	-0.02	0.80
9	20	2	1.02	0.94	-0.04	0.90
10	20	1	0.99	0.91	-0.02	0.89
11	20	1	0.95	0.87	-0.02	0.85
12	20	2	0.99	0.91	-0.04	0.87
13	21	1	0.82	0.72	-0.02	0.70
14	21	2	0.98	0.88	-0.04	0.84
15	21	1	0.92	0.82	-0.02	0.80

TABLE XIII—*concl'd.*

Serial No.	Age in years.	Skin and subcutaneous tissue thickness in mm.	C index.	C index with age correction.	Adiposity correction.	Myotrophic index.
16	22					
17	22	2	1.01			
18	22	1	0.97	0.89	-0.04	
19	23	2	0.95	0.85	-0.02	0.85
20	23	1	1.02	0.83	-0.04	0.83
21	24	1	1.03	0.90	-0.02	0.79
22	24	2	1.06	0.91	-0.02	0.88
23	25	3	1.09	0.94	-0.04	0.89
24	25	3	1.06	0.97	-0.04	0.90
25	26	1	0.95	0.94	-0.06	0.91
26	26	1	0.96	0.83	-0.06	0.88
27	27	1	1.10	0.79	-0.02	0.81
28	28	3	0.99	0.93	-0.02	0.77
29	28	1	0.99	0.82	-0.02	0.91
30	29	3	0.99	0.82	-0.06	0.76
31	29	1	1.09	0.92	-0.02	0.80
32	29	2	1.25	1.08	-0.06	0.86
33	29	3	1.07	0.90	0.02	1.06
34	29	7	0.98	0.81	0.04	0.86
35	31	0.5	1.04	0.87	0.06	0.75
36	32	2	0.96	0.79	0.14	0.73
37	33	1	0.99	0.82	-0.00	0.79
38	38	3	0.87	0.70	-0.02	0.80
39	45	4	1.08	0.91	-0.02	0.68
		3	1.06	0.89	-0.06	0.85
			1.32	1.15	-0.08	0.81
					-0.06	1.09

A Myotrophic Index.

TABLE XIV.

Age, thickness of the skin and subjacent tissue, C index, C index with age correction, adiposity correction and the myotrophic index of each person who was a beginner in taking exercise.

Serial No.	Age in years.	Skin and subcutaneous tissue thickness in mm..	C index.	C index with age correction.	Adiposity correction.	Myotrophic index.
40	14	10	1.10	1.10	0.20	0.90
41	14	3	0.78	0.78	0.06	0.72
42	16	4	0.99	0.98	0.08	0.90
43	17	4	0.90	0.88	0.08	0.80
44	18	2	0.92	0.88	0.04	0.84
45	19	7	1.00	0.94	0.14	0.80
46	19	2	0.85	0.79	0.04	0.75
47	20	3	0.76	0.68	0.06	0.62
48	20	4	1.01	0.93	0.08	0.85
49	21	5	1.07	0.97	0.10	0.87
50	22	14	1.46	1.32	0.28	1.04
51	22	0.5	0.78	0.66	0.00	0.66
52	23	0.5	0.90	0.78	0.00	0.78
53	24	3	1.03	0.91	0.06	0.85
54	24	3	0.96	0.84	0.06	0.78
55	25	5	1.14	1.02	0.10	0.92
56	26	2	0.93	0.76	0.04	0.72
57	26	2	0.86	0.69	0.04	0.65
58	27	2	0.86	0.69	0.04	0.65
59	28	3	0.90	0.73	0.06	0.67
60	30	16	1.35	1.18	0.32	0.86
61	34	10	1.11	0.94	0.20	0.74
62	40	14	1.22	1.05	0.28	0.77

TABLE XV.
Comparative distribution of the thickness
of the skin and the subjacent tissue
in exercising and non-exercising
groups.

Thickness in mm.	Exercising group.	Non-exercising group.
Up to 0.5	2	2
1	18	...
2	10	5
3	7	5
4	1	3
5	...	2
6
7	1	1
8
9
10	...	2
11
12
13	...	2
14
15
16	...	1
TOTAL ...	39	23

Average for the exercising group ... 1.82 mm.

Average for the non-exercising group ... 5.13 mm

TABLE XVI.

*Comparative distribution of the C index
with the age correction for the exer-
cising and non-exercising
groups.*

C index with age correction.	Exercising group.	Non-exercising group.
0.65—0.69	...	4
0.70—0.74	2	1
0.75—0.79	2	3
0.80—0.84	8	1
0.85—0.89	8	3
0.90—0.94	11	4
0.95—0.99	5	2
1.00—1.04	...	1
1.05—1.09	2	1
1.10—1.14	...	1
1.15—1.19	1	1
1.20—1.24
1.25—1.29
1.30—1.34	...	1
TOTAL ...	39	23

Average C index with age correction :

Exercising group ... 0.891

Non-exercising group ... 0.896

TABLE XVII.

Comparative distribution of myotrophic index in exercising and non-exercising groups.

Myotrophic index.	Exercising group.	Non-exercising group.
0.60-0.64	...	1
0.65-0.69	1	4
0.70-0.74	2	3
0.75-0.79	5	3
0.80-0.84	10	4
0.85-0.89	10	5
0.90-0.94	5	2
0.95-0.99	3	...
1.00-1.04	1	1
1.05-1.09	2	...
TOTAL	39	23
Average myotrophic index :		
Exercising group	...	0.86
Non-exercising group	...	0.79

SUMMARY.

1. Product of stature, foot-length and pelvic-width in cm. is nearly double the body-weight in grammes.
2. The values of the C index which are representative of the relative body-bulk up to the age of 22 years lie in a parabolic curve.
3. The values of C index march on in a unidirectional manner from thin type to plump type of individuals.
4. In non-obese persons the measurement of thickness of skin and subcutaneous tissue at the anterior axillary fold ranges up to 5 mm. only. In individuals of ages lower than 15 years, the cutaneous thickness in non-obese may be up to 5 mm. In non-obese individuals above the age of 15 years this thickness is only up to 3 mm.

5. Every millimetre increase in thickness of the skin and subcutaneous tissue at the anterior axillary fold is associated with the increase of C index by 0.02.

6. If the increase of 0.02 per millimetre thickness of the skin and cutaneous tissue at the anterior axillary fold is deleted from the general C index, the remainder is likely to serve as an index of the muscular bulk of a person.

7. The C index with an age correction (Table IX) and with an adiposity correction can serve as a myotrophic index, which in exercising bodies was found to be 0.86 and in non-exercising ones 0.79.

While giving an account of Franzen's attempts of finding a suitable nutritional index, Sankaran (*loc. cit.*) has stated that physicians apparently try 'to compare persons in muscle size, weight in subcutaneous tissue in relation to their skeletal measurements'. He further states that 'the theory accepted for future work was: Measurement of growth and nutritional status involve the consideration of weight, amount of muscle, and the amount of subcutaneous tissue, each taken in relation to normal expectancy of their qualities for given skeletal conformation'. The myotrophic index suggested by the author, as will have been seen, is based on consideration of weight as related to skeleton with an age correction and an adjustment for subcutaneous tissue.

CONCLUSIONS.

An urgent need has long been felt, of a somatometric index which is reliable and convenient of application, in assessment of nutritional status of persons.

Clarke (1939) in his address on future of physical anthropology remarks:—

'The problem of human genetics and the supposed association of mental traits with demonstrable physical characters, are complicated by the growing recognition during recent years that nutrition plays a highly important part in determining the physical variation seen in different races and different individuals. It is unnecessary to state that the broad relation between the nutrition and physique has always been accepted by anthropologists. Stature, weight, chest dimensions and so forth can all be correlated with nutrition. But the suggestion is now put forth by competent dietitians that the effects of malnutrition may be much more extensive, a casual agent not only underlying many types of variation in physical characters, but also in determining susceptibility of different types of infections. If this is true, then it is undoubtedly to be true that it clearly demands the serious attention of physical anthropologists'.

A widespread malnutrition is a matter of national interest and it should be corrected as soon as possible. Anthropometric survey on a large scale is required to be done. For the success of such surveys, there should be available a suitable somatometric method to assess nutrition which is reliable and convenient of application.

It will be observed from this account that the myotrophic index suggested by the present author is based on a conception which combines Franzen's idea of a nutritional status and Karl Pearson's three dimensional plea.

The state of good nutrition can hardly be expressed in a small single sentence, as in the conception of a nutritional status three distinct factors are required to be taken into consideration. These are:—

1. Weight/skeleton relationship.
2. Age factor.
3. Adipose-tissue factor.

1. Weight/skeleton relationship must involve four measurements. Body-weight and three linear measurements of the skeleton representing three dimensions.

2. Age factor is an important one and cannot be ignored as the activities of the human body normally are of different intensity at different ages, and body-forms at different ages vary.

3. A correction for adiposity is required to be done, if the quality of the tissues making up the body-bulk is required to be taken into consideration during an assessment of nutrition.

It is inevitable, therefore, that an efficient somatometric index of nutrition would become elaborate and slightly complicated.

In the opinion of the author the results of this investigation justify a hope that further work along this line may lead to the solution of an efficient somatometric index of nutrition. Further work would consist of at first evolving a caliper which would exert a standard constant pressure on the fold of the skin and subcutaneous tissue while measuring its thickness. With this standard instrument personal errors can be eliminated and the adiposity value in terms of the C index can be revised and fixed as a permanent standard.

The author must acknowledge here, with gratefulness, the help which he received from Dr. Jivraj N. Mehta, the Ex-Dean of Seth G. S. Medical College, Bombay, for providing him with sufficient funds to have an assistant while this investigation was being carried out.

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APPENDIX.

Structures of the nutritional indices suggested by different workers.

The list of the nutritional indices contrived by the various workers is given below for structural comparison with the C index. The list is compiled from the accounts of Jones (*loc. cit.*) and Sankaran (*loc. cit.*).

1. In 1829 Buffon proposed the following formula :—

$$\frac{\text{Weight}}{\text{Height}^3}$$

2. In 1836 Quetelet proposed three formulæ :—

$$(a) \frac{\text{Weight}}{\text{Height}^2}$$

$$(b) \frac{\text{Weight}}{\text{Height}^{2.5}}$$

$$(c) \frac{\text{Weight}}{\text{Height}}$$

3. In 1899 Livi proposed four formulæ :—

$$(a) \frac{100 \times \sqrt[3]{\text{Weight}}}{\text{Height}}$$

$$(b) \frac{\text{Weight}}{\text{Chest circumference}}$$

$$(c) \frac{\text{Weight}}{\text{Height} \times \text{Chest circumference}}$$

$$(d) \frac{\text{Weight}}{\text{Height} \times \text{Chest circumference}^2}$$

4. In 1908 Rohrer suggested the following formula :—

$$\frac{100 \times \text{Weight in g.}}{\text{Height in cm.}^3}$$

5. In 1909 Bobbit suggested the following formula :—

$$\frac{\text{Height}^3}{\text{Weight}}$$

6. In 1886 Bernhardt proposed various formulæ :—

$$(a) \frac{\text{Height in cm.} \times \text{Chest in cm.}}{240} = \text{Weight in kg.}$$

$$(b) \frac{\text{Height in cm.} \times \text{Chest in cm.}}{294} = \text{Weight in kg.}$$

$$(c) \frac{\text{Weight}}{\text{Height} \times \text{Chest} \times \text{Constant}}$$

$$(d) \frac{\text{Weight}}{\text{Height} \times \text{Chest}^2}$$

$$(e) \frac{\text{Weight}}{\text{Chest}}$$

7. In 1901 Pignet evolved a formula :—

Height-breast circumference in cm. \times Weight in kg.

8. In 1917 Tuxford offered the following formula for boys and girls :—

$$(a) \text{ Boys : } \frac{\text{Weight in g.}}{\text{Height in cm.}} \times \frac{381 - \text{Age in months}}{54}$$

$$(b) \text{ Girls : } \frac{\text{Weight in g.}}{\text{Height in cm.}} \times \frac{384 - \text{Age in months}}{48}$$

9. Broca's formula :—

$$\text{Height in cm.} - 100 = \text{Weight in kg.}$$

10. van Noorden's formula :—

$$(a) \text{ Height in cm.} \times 0.455 = \text{Weight in kg.}$$

$$(b) \text{ Height in cm.} \times 0.214 = \text{Weight in kg.}$$

11. Bardeen's formula :—

$$\frac{\text{Weight in lb.}}{\text{Height in inches}^3} \times 1,000$$

12. Bruysch's formula :—

$$\frac{\text{Chest}}{\text{Height}}$$

13. Brailsford's formula :—

$$(a) 100 \times \sqrt[3]{\text{Weight}}$$

$$(b) \frac{100 \times \text{Weight}}{\text{Height}}$$

14. von Pirquet's formula :—

$$\text{Pelidici} \frac{\sqrt[3]{\text{Weight} \times 10}}{\text{Sitting height.}}$$

15. A.C.H. index of American Child Health Association.

Five readings are needed for the determination of this index :—

Arm circumference in (1) flexed, (2) relaxed condition.

Chest antero-posterior depth in (3) expiration, (4) inspiration.

Hip-width between trochanters (5).

The following body-parts seem to have been utilized for nutritional indices so far :—

1. Height.
2. Chest circumference.
3. Antero-posterior depth of the chest.
4. Arm circumference.
5. Hip-width.
6. Distance between the two trochanters.
7. Sitting height.
8. Body-weight.

NICOTINIC-ACID REQUIREMENTS OF INDIAN ADULT.

BY

H. N. DE,

AND

K. C. BANERJEE.

(From the Nutrition Research Unit, I. R. F. A., Biochemical Laboratory,
Dacca University, Dacca.)

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IN our attempts to evaluate the requirements of the different vitamins by normal Indian adult, the first work of the series—'Ascorbic-acid requirement of Indian adult'—investigated in this Laboratory has been reported previously by De and Chakravorty (1948). The present communication deals with the nicotinic-acid requirement of Indian adult by metabolic study with poor rice diet.

The study of the urinary response to the above vitamin at graded doses of intake by normal individual is also reported in the present work.

Very few investigations in this line are reported in the literature. Sarett *et al.* (1942) and Briggs *et al.* (1945) have made attempts to determine the nicotinic-acid requirement of human adult by urinary response method. Swaminathan (1939), Melnick *et al.* (1940) and Johnson *et al.* (1945) have studied the recovery of the ingested dose of nicotinic acid in human adult urine but their results showed great discrepancy.

EXPERIMENTAL AND RESULTS.

A. Nicotinic-acid tolerance test with rice diet.

Sarett *et al.* (*loc. cit.*) in their study of the nicotinic-acid requirement of man suggested a tolerance test in which the extra excretion of less than 5 per cent of the test dose of 500 mg. was regarded as indicative of nicotinic-acid deficiency. These workers have not reported the dietary intake of nicotinic acid of the subjects on whom the saturation test experiment was conducted. Briggs *et al.* (*loc.*

cit.), by carrying out the same test on two adults living on restricted diet containing about 3 mg. of nicotinic acid, contradicted the above findings of Sarett *et al.* (*loc. cit.*) and expressed the view that anything less than 15 per cent of the extra excretion should be considered as representing deficiency. They also suggested that the intake of 3 mg. of nicotinic acid by the subjects was somewhere near the minimal human requirement.

For the study of the nicotinic-acid requirement of Indian adult by tolerance test the above technique of Briggs *et al.* (*loc. cit.*) was adopted. The test was conducted on five normal adults subsisting on rice diet composed of 550 g. to 600 g. parboiled milled rice, 70 g. dhal, 50 g. fish, 200 g. vegetables and 30 g. oil. After a collection period of seven days on the basal diet the test dose of 500 mg. of nicotinic-acid amide was given daily for four days and the extra excretion due to test dose was estimated. The basal rice diet furnished 7.2 mg. to 9.6 mg. of nicotinic acid daily. The technique of collection of urine was the same as in the previous paper from this Laboratory (De and Chakravorty, *loc. cit.*).

The nicotinic-acid content of foodstuffs and urine was estimated by the methods of Swaminathan (1942) and Wang and Kodicek (1943). The urinary excretion represents nicotinic acid, nicotin amide, nicotin uric acid and about 35 to 40 per cent of trigonelline, and all these have been expressed as total nicotinic acid of urine.

TABLE I.

Urinary response of nicotinic acid on tolerance test dose of 500 mg. nicotin amide.

Subject.	Dietary intake in mg.	Average daily excretion on basal diet.	TOLERANCE TEST DOSE—500 MG.	
			Average daily excretion.	Extra excretion as per cent of test dose.
B. S. : body-weight—49 kilos. ...	7.8	4.9	115.0	22.2
M. R. : body-weight—48 „ ...	7.2	3.7	109.2	21.1
A. D. : body-weight—45 „ ...	8.4	6.2	108.2	20.4
B. M. : body-weight—53 „ ...	9.6	5.1	102.1	19.4
S. T. : body-weight—46 „ ...	8.2	7.9	121.9	22.8
Average ...	8.2	5.5	111.5	21.2

The results of the tolerance tests are presented in Table I from which it will be observed that the average intake and excretion of nicotinic acid of the five adults on rice diet were 8.2 mg. and 5.5 mg. respectively and the mean total excretion on 500 mg. test dose was found to be 111.5 mg. The average value of the extra excretion due to test dose was calculated to be 21.2 per cent and since this value was more than the minimum limit for deficiency as suggested by Briggs *et al.* (*loc. cit.*), the above rice diet containing 7.2 mg. to 9.6 mg. of nicotinic acid may be regarded as sufficient to supply the nicotinic-acid requirement of an Indian adult. The present work supports the observations of Aykroyd and Swaminathan (1940) who in their dietary surveys found pellagra rare even among impoverished population living on rice diet which supplied 5 mg. to 11 mg. nicotinic acid depending on the type of the rice used. The daily allowance of 15 mg. of nicotinic acid, as recommended by the National Research Council, seems to be somewhat higher when judged in the light of the present investigation.

B. Urinary response of nicotinic acid at various doses of intake.

The urinary response of nicotinic acid in human adult at different levels of intake has also been investigated. Melnick *et al.* (*loc. cit.*) showed that at a lower level of intake of 3.4 mg. per kg. body-weight, a small quantity as 3 to 4 per cent of pyridine compounds was excreted in human urine but as the dose increased to 9.4 mg. per kg. body-weight, there was augmentation of extra excretion from 14 to 25 per cent. Johnson *et al.* (*loc. cit.*) found recovery of 3 per cent of the ingested nicotinic acid in human urine. Swaminathan (1939) in the course of his studies of the point of saturation with nicotinic acid in one man living on rice-milk diet showed that 13 per cent of the intake on an average was daily excreted in urine irrespective of the dose and of the period of administration. This part of the work has been taken up to get more precise information about urinary response of nicotinic acid in human subject under different doses.

Five normal adults, on whom the tolerance test was previously conducted, were also employed in this part of the investigation after an interval of six weeks. The diet employed was the same as that used in the tolerance test. After a period of eight days on basal diet the subjects were given daily dose of nicotinic-acid amide varying from 50 mg. to 400 mg. Each dose was continued for eight days and the collection of urine was made on the last four days of the period. The results presented in Table II show that at doses of 50 mg., 100 mg., 300 mg. and 400 mg. of nicotinic-acid amide the extra excretions of the acid were 7.3, 14.7, 21.0 and 22.1 per cent, respectively. It is also evident from the data that as the dose was doubled from 50 mg. to 100 mg. the extra excretion also increased in a similar proportion from 7.3 per cent to 14.7 per cent and when the daily dose was increased to 300 mg. there was further augmentation of the extra excretion to 21.0 per cent. As the dose was further increased to 400 mg., the extra excretion did not show any definite change but remained in the region of 22.1 per cent. In the first part of the experiment dealing with the tolerance test, it was also observed that at the dose of 500 mg. of nicotinic-acid amide the extra excretion was in the region of 21.2 per cent. Since the extra excretions due to 300 mg., 400 mg. and 500 mg. doses were almost of the same order, it may be interpreted that the body most probably became saturated at 300 mg. test dose.

TABLE II.

Human urinary response of nicotinic acid at varying doses of intake.

Experimental subject.	Dietary intake in mg.	Average daily excretion in mg. on basal diet.	Period II. Dose—50 mg. N. A.		Period IV. Dose—100 mg. N. A.		Period VI. Dose—300 mg. N. A.		Period VIII. Dose—400 mg. N. A.	
			Average daily total excretion in mg.	Extra excretion as per cent of test dose.	Average daily total excretion in mg.	Extra excretion as per cent of test dose.	Average daily total excretion in mg.	Extra excretion as per cent of test dose.	Average daily total excretion in mg.	Extra excretion as per cent of test dose.
B. S.	7.2	4.6	9.8	10.4	21.4	16.8	74.2	23.2	95.4	22.7
M. R.	8.3	4.2	8.7	9.0	20.8	16.6	70.2	22.0	96.7	23.1
A. D.	7.8	4.8	8.7	7.8	17.8	13.0	64.3	19.9	92.1	21.8
B. M.	8.8	6.8	8.9	4.2	18.3	11.5	65.9	19.7	87.6	20.2
S. T.	8.1	7.2	9.8	5.2	23.0	15.8	68.4	20.4	97.9	22.7
Average ...	8.0	5.5	9.2	7.3	20.3	14.7	68.6	21.0	93.9	22.1

N. A.—nicotinic-acid amides ('Benedict'—Roche). Acknowledgment is made to Volkart Bros. for generous supply of this product. The periods which are not indicated in the table should be regarded as preliminary periods.

SUMMARY.

1. By tolerance test on five normal human adults it has been observed that 7.2 mg. to 9.6 mg. of nicotinic acid present in rice diet is sufficient to supply the Indian adult requirement.

2. The study of the urinary response of various doses of nicotinic-acid amide has indicated that the per cent of extra excretion at low doses of intake is lower than that at higher doses. The value gradually increases with the increase in dose until it reaches a maximum limit beyond which it cannot exceed, irrespective of further administration of higher doses.

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STUDIES ON THE EFFECT OF NICOTINIC ACID ON
THE BLOOD SUGAR AND URINARY EXCRETION
OF SUGAR OF NORMAL AND
DIABETIC RABBITS.

BY

SACHCHIDANANDA BANERJEE, M.B., D.Sc. (Cal.), M.A.D.A. (U.S.A.),
NARESH CHANDRA GHOSH, M.Sc..

AND

GANGAGOBINDA BHATTACHARYA, M.Sc.
(From the Department of Physiology, Presidency College, Calcutta.)
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SINCE nicotinic acid as part of the complex enzymes, co-enzyme I and co-enzyme II is involved in carbohydrate metabolism, its function in the treatment of diabetes has attracted considerable attention. In a group of 94 diabetics studied by Biskind and Schreier (1945), every one showed signs and symptoms of deficiency in factors of B vitamins. Glossitis occurred in almost all the patients. Chiliosis, gastro-intestinal disturbance, nervousness and polyneuritis were frequently noticed. All these conditions showed prompt response to intensive nutritional therapy. Rudy and Hoffmann (1942) observed that skin disturbances common in diabetes mellitus appeared to be caused by a deficiency in the components of the vitamin-B complex, especially nicotinic acid. The lesions usually responded to treatment with nicotinic acid. Sydenstricker *et al.* (1939) noted glossitis, chiliosis and stomatitis in diabetics that cleared up with nicotinic acid. Göbell (1941) reported a fall in the blood-sugar level when 100 mg. of nicotinic acid were intravenously injected in children. Marche and Delbarre (1943), Poumeau-Delille and Fabiani (1943) and Neuwahl (1943) also reported the hypoglycæmic action of nicotinic acid in normal human adults. Gordon (1946) treated a case of diabetes

by feeding 1,200 mg. of nicotinic-acid amide per day for one month. The fasting blood sugar dropped down to 60 mg. per 100 c.c. blood from the initial value of 160 mg. per 100 c.c. blood. Ledrut (1940) reported a marked decrease in the blood-sugar level in normal rabbits which received nicotinic acid either orally or intravenously. Contrary to the above findings, Horst (1941) observed that injection of nicotinic-acid amide to persons with normal metabolism had no influence on the fasting blood-sugar level or on the glucose tolerance. Banerjee (1947) reported that injection of nicotinic acid along with the injection of alloxan prevented the development of diabetes in rabbits and rats. Banerjee has suggested that if diabetes is produced by the imperfect metabolism of purines whereby alloxan is produced in the system, nicotinic acid might play some part in the prevention of the development of diabetes. The effect of nicotinic acid on the blood sugar and urinary excretion of sugar was, therefore, studied in normal and diabetic rabbits and rats. Urinary excretion of nicotinic acid was also studied in rabbits before and after the development of diabetes.

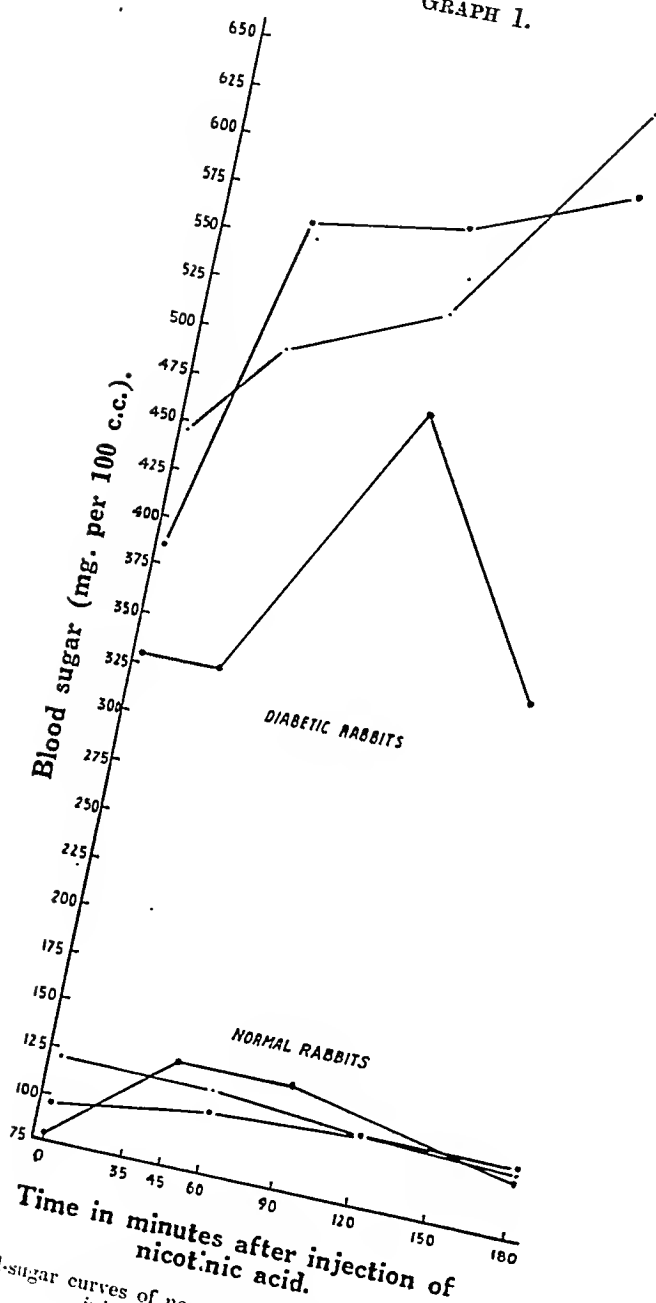
EXPERIMENTAL.

(a) *Effect of nicotinic acid on the blood-sugar level in normal and diabetic rabbits.*—Six healthy male Himalayan rabbits of weights varying between 1,400 g. and 1,700 g. were used. Three of the rabbits were made diabetic by injection of alloxan as follows: A 10 per cent solution of alloxan in water was injected into the marginal ear vein of each rabbit in the dose 200 mg. per kilo body-weight of the animal. Initial hypoglycæmia was prevented by several intravenous injections of 20 c.c. of a 50 per cent solution of glucose. Each animal showed persistent hyperglycæmia and glycosuria on the following day and developed diabetes. Three days after the injection of alloxan, all six animals were fasted for 24 hours. After a fasting blood sample was taken from the marginal ear vein, 2.5 c.c. of a 2 per cent solution of nicotinic acid (50 mg.) were injected intravenously and samples of blood were taken at fixed intervals up to 180 minutes thereafter. Blood sugar was determined according to the method of Hagedorn and Jensen (1923). Curves are shown in Graph 1.

(b) *Effect of nicotinic acid on glucose-tolerance curves of normal rabbits.*—Four healthy male rabbits were starved for 24 hours. After the fasting-blood samples were taken, all of the rabbits received an intravenous injection of a 50 per cent solution of glucose (1 g. per kilo). Samples of blood were taken at intervals of 45 minutes up to 225 minutes after the injection of the glucose. Blood sugar was determined as before. Three days after, the glucose tolerance was repeated in the above four rabbits with the modification that immediately after the injection of glucose all the four rabbits were given 50 mg. of nicotinic acid, also by intravenous injection. The tolerance curves are shown in Graph 2.

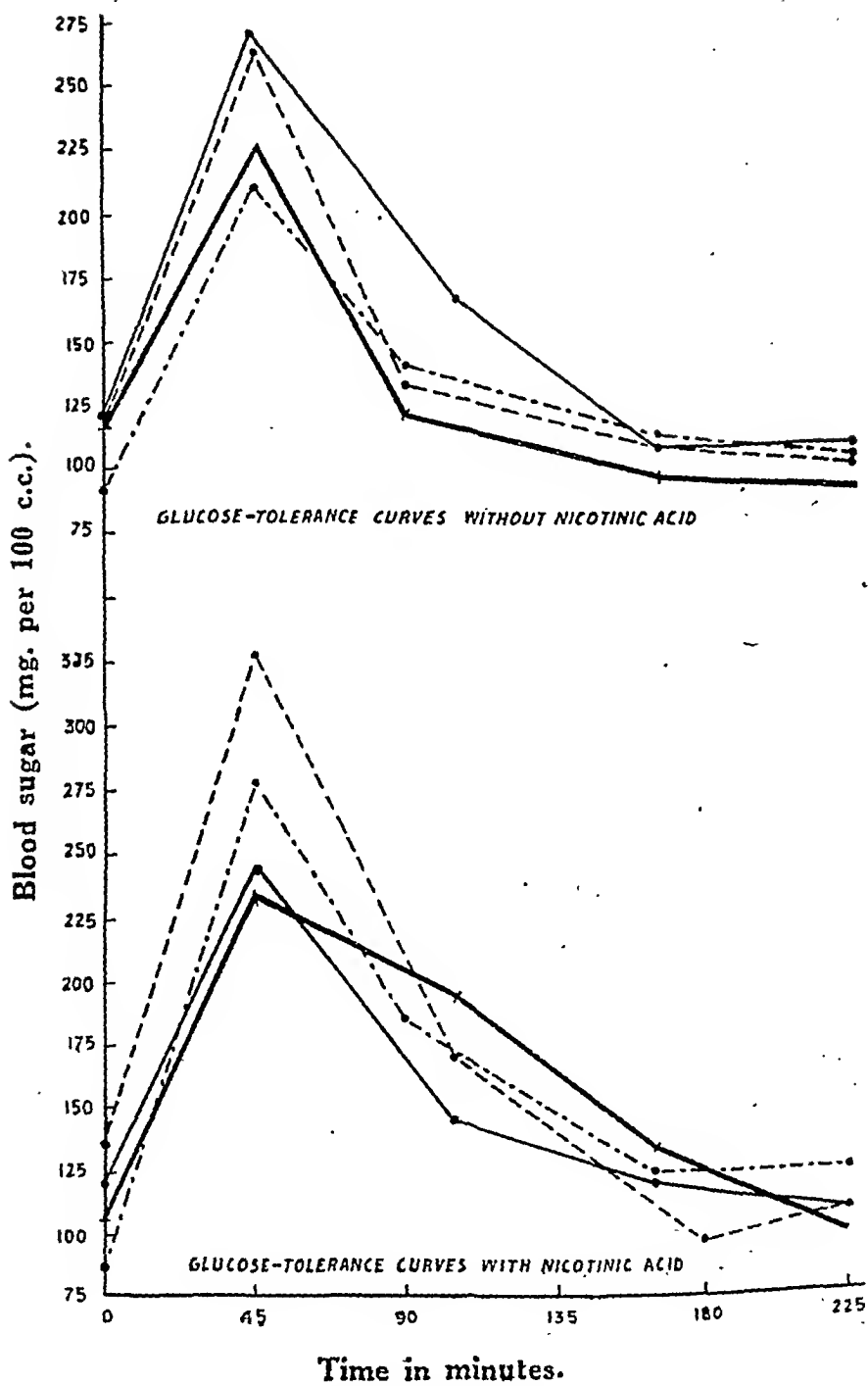
(c) *Effect of nicotinic acid on the urinary excretion of sugar by diabetic rabbits.*—Three rabbits made diabetic by intravenous injection of alloxan (200 mg. per kilo) were placed in individual metabolism cages and their urine collected under toluene. Urinary sugar was determined with Benedict's quantitative reagent. Each animal then received an intramuscular injection of 50 mg. of nicotinic acid in a 2 per cent

GRAPH 1.



Blood-sugar curves of normal and diabetic rabbits after injection of nicotinic acid.

GRAPH 2.



Glucose-tolerance curves of rabbits with and without nicotinic acid.

solution, the urine was again collected for 24 hours and the sugar was estimated as before. The results are shown in Table I:—

TABLE I.
*Twenty-four-hour urinary excretion of
sugar by diabetic rabbits before
and after injection of 50 mg.
of nicotinic acid.*

Rabbit number.	Before (g.).	After (g.).
1	2.6	2.2
2	5.7	5.3
3	5.8	5.7

(d) *Effect of alloxan diabetes on the urinary excretion of nicotinic acid.*—Eleven rabbits were fed on a diet consisting of germinated gram (*Cicer arietinum*). They were placed in separate metabolism cages and their urine was collected over 1 c.c. concentrated hydrochloric acid. Nicotinic acid was estimated chemically by the method of Banerjee *et al.* (1948). The animals were then made diabetic by alloxan (200 mg. per kilo) and the urinary excretion of nicotinic acid was then determined for another 2 days. The results are shown in Table II:—

TABLE II.
*Twenty-four-hour urinary excretion of nicotinic acid by rabbits
before and after making them diabetic with alloxan.*

Rabbit number.	BEFORE :		AFTER :	
	First day (γ)	Second day (γ)	First day (γ)	Second day (γ)
1	195	150	156	164
2	195	136	150	189
3	145	146	182	Died.
4	213	412	398	308
5	222	163	280	Died.
6	176	252	193	212
7	115	333	328	217
8	...	550	752	954
9	230	200	354	330
10	203	170	254	Died.
11	...	490	512	...

RESULTS.

Nicotinic acid did not produce any appreciable change in the blood sugar of two of the normal rabbits, but in the third a slight rise occurred. No hypoglycæmia was noted. In three diabetic rabbits injection of nicotinic acid caused an increase in the blood-sugar level which in two of the rabbits went on rising during the 180 minutes studied. In the third rabbit the blood-sugar level came down after 90 minutes but remained higher than the fasting level even after 180 minutes. Injection of nicotinic acid did not alter the glucose-tolerance curves. Urinary excretion of sugar by the diabetic animals was not appreciably altered after the injection of nicotinic acid and urinary excretion of nicotinic acid did not vary much when diabetes developed.

DISCUSSION.

The observation that nicotinic acid had no influence either on the blood sugar or on the glucose tolerance of normal rabbits does not support the claims of Göbell (*loc. cit.*) and others. Neuwahl (*loc. cit.*) and Göbell (*loc. cit.*) concluded that the action of nicotinic acid is similar to that of insulin. However, the injection of nicotinic acid to the diabetic rabbits not only did not reduce the fasting blood sugar but caused a marked and prolonged increase in the blood-sugar level. Unlike injections of insulin, the injection of nicotinic acid had no influence on the urinary excretion of sugar by diabetic rabbits. Had nicotinic acid acted like insulin, there would have been a greater demand for it when the animal developed diabetes, and consequently less nicotinic acid would have been excreted in the urine. Though the animals received the same diet throughout the experimental period, the urinary excretion of nicotinic acid was not much altered when the animals developed diabetes. Nicotinic acid does not act like insulin; instead of ameliorating the diabetic condition, it only enhanced it.

SUMMARY.

1. Nicotinic acid had practically no effect on the blood sugar of normal rabbits but it raised considerably the blood sugar of alloxan diabetic rabbits.
2. It showed no effect on glucose tolerance in normal rabbits.
3. Nicotinic-acid excretion did not diminish when the rabbits developed diabetes.
4. Nicotinic acid did not cause the urinary excretion of sugar by diabetic rabbits to diminish.
5. Nicotinic acid does not act like insulin and it seems to have no effect in experimental diabetes.

Nicotinic acid was kindly supplied by Hoffmann la Roché, Inc., Basle, Switzerland, through Dr. T. J. Thompson Wells of Messrs. Volkart Brothers, Bombay.

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FORTIFICATION OF SOYA-BEAN MILK WITH CALCIUM AND STUDY OF ITS AVAILABILITY TO YOUNG GROWING RATS.

BY

B. T. KARNANT,

S. S. DE,

AND

V. SUBRAHMANYAN.

(From the Department of Biochemistry, Indian Institute of Science, Bangalore.)
(An Inquiry under the Indian Research Fund Association.)

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IN earlier communications, evidence has been adduced (Desikachar, De and Subrahmanyan, 1946, 1948; Sahasrabudhe, Desikachar and Rao, 1947) to show how the digestibility and biological value of soya proteins are raised to practically the same level as that of cow's milk by the methods of processing employed for the preparation of the milk. The vitamins of the B group as present in the processed soya milk and in cow's milk have also been compared and found to be of nearly the same order. Soya milk has a supplementary value when added to the poor cereal diet though the bean when fed as a whole (raw or cooked) has practically no such value (Aykroyd and Krishnan, 1936, 1937, 1937a; Report of the Soya-bean Sub-Committee, I.R.F.A., 1946).

Although soya milk is fairly adequate in other respects, it is nevertheless deficient in calcium (Ca 20 mg. in 100 c.c.) as compared with cow's milk (Ca 120 mg. in 100 c.c.). Apart from its general importance, calcium is of special significance in the dietary of vegetarians and particularly as a supplement to the poor South Indian rice diet which is highly deficient in that element. Earlier investigations by Aykroyd and Krishnan (*loc. cit.*), Basu *et al.* (1939, 1941) and others have shown that the supplementary effect of cow's milk when added to the poor rice diet is largely due to its calcium content. Desikachar *et al.* (1946) have shown that the supplementary value of plain soya milk to the poor rice diet is 60 per cent that of cow's milk. In view of the earlier observations, it appeared probable that soya milk suitably fortified with calcium will not only have a higher supplementary value, but also possess a greater overall food value than the unfortified product.

The present investigation was accordingly taken up with the following objects: (i) to standardize conditions for the incorporation of such calcium compounds as would not interfere with the flavour, palatability and stability of milk; (ii) to investigate the extent of availability of calcium and phosphorus from the fortified milk; and (iii) to compare the nutritive value of fortified soya milk with that of cow's milk by the study of the growth response of young rats.

EXPERIMENTAL.

General plan.—The experiment was designed as a complete metabolic study of sufficient duration to obtain a balance of calcium by measuring the total intake of calcium and its excretion in urine and faeces. As a check on the accuracy of the technique the calcium contents of the experimental rats at the end of the series were compared with those of their litter mates at the beginning of the experiment. The deposition of calcium thus determined should fairly closely tally with the difference between the intake and excretion of calcium by the experimental animals.

Animals and diet.—Animals of the same litter, four weeks old and weighing about 40 g. each, were taken for the metabolic studies. The animals were given calculated quantities of the basal diet supplemented with soya milk fortified with calcium as described later. The calcium content of the milk and the diet were determined on average samples collected from day to day. Weighings and other systematic observations were made once in three days.

Basal diet—low calcium and phosphorus diet.

	Per cent.
Dried, heat coagulated, egg-white* from new-laid eggs	... 14.25
Calcium- and phosphorus-free salt mixture	... 2.85
Cane sugar	... 9.6
Maize starch	... 68.3
Butter fat	... 5.0

This diet was found to contain 0.02 per cent calcium and 0.025 per cent phosphorus.

Vitamins.—As sources of vitamins A and D the experimental rats were given one drop of shark-liver oil (100 I.U.) every alternate day and calciferol (30 I.U.) twice a week. Yeast tablets (Squibbs) were given at the rate of half a tablet per rat per day to ensure adequate supply of vitamin of the B complex.

Method of feeding.—The basal diet was offered slightly in excess of the intake. If only small amounts were left unconsumed, the same quantity of fresh diet was added each day. In case fairly large quantities were left unconsumed, fresh additions were regulated in accordance with the actual consumption.

Collection of urine and faeces.—These were done in accordance with the usual procedure for metabolic studies.

* The whites of eggs were carefully separated, coagulated by heating in a double saucepan for a few minutes and then dried in the oven at 60°C.

METHODS OF ANALYSIS.

Milk.—Twenty c.c. of milk were pipetted daily into a silica dish and evaporated to dryness on the water-bath. Mixed samples (3 to 5 days) were ashed and analysed for calcium (McCrudden, 1911). Phosphorus in milk was estimated by Fiske and Rao (1925) method.

Fæces.—The fæces from each rat were placed in a silica dish and 5 c.c. of sodium acetate (10 per cent) were added. After evaporating to dryness, the fæces were ashed, and calcium and phosphorus estimated by the same method.

Urine.—The urine collected with 5 per cent nitric acid was first concentrated to one-fourth of its volume. It was then oxidized according to the method of Shohl and Pedley (1922) with ammonium persulphate, then filtered and made up to volume. Calcium and phosphorus were estimated by the methods already cited.

Incorporation of calcium in the milk.—It was found that more than minute amounts of calcium in any form could not be added to soya milk without the risk of clotting on subsequent heating unless the reaction was suitably adjusted. There was also marked difference in regard to the extent to which each calcium salt could be incorporated. There was practical difficulty in the addition of the stronger alkalis as they often tended to pass the required limit and also to impart an undesirable taste and flavour to the milk. It was found desirable, therefore, to use sodium bicarbonate for adjusting the reaction. Whenever possible, the calcium salt was prepared in the form of a solution and added in small instalments till the limit of stability was reached. Before incorporating any further quantity, the reaction was adjusted by addition of bicarbonate and fresh calcium then added. The maximum concentration aimed at was 120 mg. of calcium in 100 c.c. but it could not be attained in any of the cases. In the case of calcium carbonate and marble, known quantities of finely powdered material were added at the time of pasting the extracted soya-bean and the milk prepared in the usual way. This

TABLE I.

Fortification of soya milk with calcium using different salts of calcium.

	Calcium gluconate (8 per cent solution).	Calcium lactate (10 per cent solution).	Calcium glycero- phosphate.	Marble.	CaCO ₃
The maximum amount of calcium salt that can be incorporated (100 c.c. of soya milk in each case).	6.0 c.c.	9.0 c.c.	133 mg.	417 mg.	167 mg.
pH adjusted ...	6.6	5.8	6.6	6.7	6.8
Calcium content per 100 c.c. after fortification.	64.1	98.6	46.0	48	46.2

TABLE II.

Availability of calcium and phosphorus in soya-bean milk.
(Fortified with calcium di-hydrogen phosphate.)

Rat number.	AVAILABILITY OF CALCIUM.				AVAILABILITY OF PHOSPHORUS.			
	CALCIUM EXCRETED IN MG.		Total calcium retained in mg.	Percentage of calcium assimilated.	PHOSPHORUS EXCRETED IN MG.		Total phosphorus retained in mg.	Percentage of phosphorus assimilated.
	Urine.	Feces.			Urine.	Feces.		
I	19.78	11.22	141.91	82.1	9.04	16.82	151.45	87.5
II	15.41	10.17	148.42	89.7	5.58	22.4	179.36	86.5
III	18.78	12.54	140.28	81.2	9.63	18.75	178.96	96.3
IV	16.84	11.54	144.52	83.6	12.25	17.6	177.49	87.3
V	15.77	21.65	135.47	78.3	10.8	10.34	186.2	89.8
VI	19.2	13.32	140.3	81.1	8.33	15.73	183.28	88.4

The period of experiment was five weeks during which each animal received 235 c.c. of soya milk. The calcium and phosphorus ingested per animal was 172.91 mg. and 207.34 mg. respectively.

resulted in the weak acids present in the processed bean reacting with the milk, the reaction being shifted towards neutral and calcium up to a certain limit going into solution. In the case of marble powder, the unused material was left in the residue after separating of milk; but in the case of calcium carbonate, there was a tendency for minute quantities of the finely divided powder to pass mechanically into the milk. Every possible care was taken in filtering the milk, but in that case some part of the carbonate may still have existed in a fine state of division.

The analytical figures show that the added calcium is not quantitatively retained in the milk. For practical purposes, we have to depend on the calcium as actually found in the final product. In the case of the soluble salts of calcium, there was always the risk of the milk curdling if the safe limit was passed even by a small margin. Repeated boiling also disturbed the stability of the milk. Furthermore, it was found that some of the salts, even at low concentrations, affected the taste and stability of the milk. By incorporating marble or calcium carbonate, a maximum of only about 50 mg. in 100 c.c. could be obtained, but the resulting milk, especially in the case of marble, had a pleasant taste and flavour. Calespar was also tried, but was found to be less reactive than the other two forms of calcium carbonate.

In our semi-large-scale preparations we have used marble or calcium carbonate. We have so far prepared about 400,000 lb. of milk and used them for school-feeding and other experiments.

In cow's milk calcium occurs in a medium that is buffered by phosphate, citrate and carbonate of alkali metals, chiefly sodium. Our experience has shown that di-calcium phosphate can be incorporated with soya milk and under proper conditions, the calcium content can be raised to about 30 mg. in 100 c.c. Pure calcium-hydrogen phosphate is, however, rather costly. It should, however, be possible to prepare a cheap product specially suited for fortification. With this object we are now trying a number of natural sources, as also commercial products, which could be adapted for use.

In the present research we considered it desirable to use specially prepared di-calcium phosphate obtained by treating pure phosphoric acid with the calculated quantity of calcium hydroxide. The preparation had a reaction of pH 6.5 and a fairly concentrated solution was added drop by drop to soya milk in such a proportion as to maintain the calcium content of the milk at 73.5 mg. per 100 c.c.

Comparative growth studies with cow's milk and calcium-fortified soya milk.—The availability of calcium from cow's milk has been studied by several previous investigators (Kon, 1929, 1931; Henry and Kon, 1937). It was not considered necessary, therefore, to carry out a fresh study on this aspect. The present studies were confined, therefore, to a comparison of the growth rates on the calcium-free diet as supplemented with cow's milk on the one hand and calcium-fortified soya milk on the other, both the milks being administered on the same volume basis (7 c.c. per day). There were six animals of each sex on each milk.

The results showed that the overall growth response was practically the same in both the cases. In the early stages, there was somewhat less response to soya than to cow's milk. This was, however, made up in the later stages. The average weekly increase in the case of cow's milk was as follows: male, 10.6 g.; female, 10.4 g. The corresponding figures for soya milk were, male: 11.0 g.; female, 10.0 g.

SUMMARY.

1. The calcium content of soya milk can be raised by incorporation of different calcium salts after suitable adjustment of reaction. Di-calcium phosphate can be added so as to raise the level of calcium to about 80 mg. per 100 c.c. of milk. The fortified product has a pleasant taste and flavour. Other calcium salts, such as gluconate, lactate and glycono-phosphate, can also be incorporated, but the phosphate generally gives a better product. Calcium carbonate has the advantage that it can be easily incorporated—though at a lower level—without any special precautions in regard to previous adjustment of reaction.

2. It was found that rats retained 82 per cent of the calcium and 87 per cent of the phosphorus ingested from fortified soya milk.

3. The growth rates of rat on a calcium-free synthetic diet supplemented with cow's milk and soya milk respectively, on the same volume basis, were practically of the same order.

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RELATIVE UTILIZATION OF CALCIUM FROM SOYA MILK (FORTIFIED WITH DI-CALCIUM PHOSPHATE) AND COW'S MILK BY GROWING CHILDREN.

BY

B. T. KARNANI,

S. S. DE,

V. SUBRAHMANYAN,

Department of Biochemistry, Indian Institute of Science, Bangalore,

AND

MISS D. CARTNER,

Lady Curzon Hospital, Bangalore.

(An Inquiry under the Indian Research Fund Association.)

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IN the previous paper (Karnani *et al.*, 1948) experiments on the incorporation of calcium in soya milk and its utilization by rats have been described. The studies showed that when calcium-fortified soya milk was used as supplement to a diet which is deficient in regard to calcium and phosphorus, the animals utilized over 80 per cent of the ingested calcium and that the growth response was practically the same as that on cow's milk. As human subjects are known to utilize calcium less efficiently than experimental animals, it was felt that the trials should be repeated under carefully controlled conditions with growing children.

Utilization of calcium from different sources by man has been studied by a number of earlier workers. It has been found that the calcium present in several green leaves is as well utilized as that in milk (Speirs, 1939), green betel leaves and pumpkin (Basu *et al.*, 1941), whereas that present in roots is used less efficiently (carrots) (Breiter, Mills, Rutherford, Armstrong and Outhouse, 1942). In a comparative study of the utilization of calcium from cow's milk and soya-bean curd by three Chinese subjects, Adolph and Chin (1932) showed that the two sources were nearly equally effective. In these studies 80 per cent of the calcium were derived from the milks.

In the present investigation, systematic metabolic studies were carried out with six children, aged 7 to 9 years, from a local orphanage. They were excellent

subjects and willingly co-operated in the rigorous discipline that had to be observed during the period of the experiment. The authorities of the orphanage were also most helpful.

The following were the general observations relating to the children :—

Subject.	Age.		Weight (lb.).	Height (inches).	General observation.
	Years.	Months.			
D. C.	7	0	39	43	The general health and nutrition of the children were normal with no marked vitamin deficiencies.
L. R.	7	4	37	44	
O. C.	8	5	44	49	
P. C.	8	2	42	47	
F. B.	8	11	48	49	
D. B.	7	1	38	43	

Prior to the beginning of experimental period, the approximate energy requirements of the experimental subjects were calculated and proper adjustment then made during the first days of the preliminary period to ensure a subjective sense of adequacy of intake. The children were then given a uniform diet which was given in weighed quantities and which they consumed *in toto* throughout the period of investigation. The main ingredients of the diet are given below :—

Experimental diet.

Foodstuff.	Amount of food consumed every day in g.
Wheat (as chapatti)	100
Bread	150
Beef	50
Rice	84
Butter	14
Vanaspati (M. P. 43°C.)	14
Brinjals	28
Tomatoes	28
Bengal gram (as dhal)	56

Experimental diet—concl.

Foodstuff.	Amount of food consumed every day in g.
Potatoes	150
Plantains (fruit)	103
Sugar	25
Vitamins (multi-vitamin pellets, B.D.H.) ...	1 tablet.*

* Each pellet contained approximately vitamin A (3,000 I.U.), vitamin B (200 I.U.), vitamin C (12.5 mg.) and vitamin D (600 I.U.).

After an initial period of ten days, the experimental period of fifteen days on the basal diet followed. This period was divided into 5-day groups for analytical purposes. In the second fifteen-day period, the basal diet was supplemented with soya milk fortified with di-calcium phosphate, while in the third period they received a supplement of cow's milk; there was no indication that the dietary régime affected the health of the subjects adversely. The changes in body-weight were insignificant.

The method followed by Steggerda and Mitchell (1939) was used in the evaluation of the efficiency of utilization of calcium in the present study. The plan was to first follow the daily loss of calcium in urine and faeces consequent on the intake of a low calcium diet. After the preliminary period, extra calcium in the form of fortified soya milk and cow's milk respectively was added to the diet and the calcium balance again determined.

Samples of the food preparations were collected periodically in proportion to the actual consumption and calcium determinations made on aliquots of pooled samples. Modification of the McCrudden (1911) method was used in the analyses of foods, faeces and urine. The faeces of successive collection periods were separated. The fresh faeces were covered with 95 per cent ethanol and dried on the water-bath. The analyses were made on aliquots of the dried and ground material. Both food and faecal analyses were carried out on samples previously flamed and ashed at 350°C. to 600°C. Twenty-four-hour urine samples were collected and aliquots preserved with hydrochloric acid and toluene. After a five-day collection period, the pooled urine samples from each subject were again adjusted to volume and aliquots removed for analysis.

Only those metabolic data which are pertinent to the subject of the utilization and retention of calcium are presented in this paper. The calcium-balance data are given in Table I. The corresponding data for calcium level and pH of blood are given in Table II. The basal diet provided an average of 22½ mg. of calcium daily on which the daily balances for the basal period varied from -135 mg. to -29 mg. The calcium excreted daily on this low calcium diet averaged 278.5 mg. of which 212 mg. were excreted by way of the intestinal tract. The daily

TABLE I.

Utilization of calcium in soya milk (fortified with di-calcium phosphate) and cow's milk based on the daily average metabolism.

Subject.	Calcium supplement tested.	Test period, days.	AVERAGE DAILY METABOLISM OF CALCIUM.					Average daily balance in mg.	Percentage utilization of supplementary Ca.
			CALCIUM INTAKE IN MG.		CALCIUM EXCRETION IN MG.		Fæces.		
			Basal diet.	Supplement.	Urine.				
D. C.	None ...	15	224	0	49	195	- 20	... 18.8	
	Soya milk	15	224	362	91	447	+ 48	... 23.3	
	Cow's milk	15	224	365	72	452	+ 65		
L. R.	None ...	15	224	0	82	184	- 42	... 27.9	
	Soya milk	15	224	362	87	440	+ 59	... 26.0	
	Cow's milk	15	224	365	79	457	+ 53		
O. C.	None ...	15	224	0	71	288	- 135	... 30.8	
	Soya milk	15	224	362	140	469	- 23	... 23.55	
	Cow's milk	15	224	365	66	572	- 49		
P. C.	None ...	15	224	0	66	220	- 64	... 19.8	
	Soya milk	15	224	362	91	487	+ 8	... 20.0	
	Cow's milk	15	224	365	53	527	+ 9		
E. B.	None ...	15	224	0	74	185	- 35	... 22.95	
	Soya milk	15	224	362	70	468	+ 48	... 19.2	
	Cow's milk	15	224	365	88	466	+ 35		
D. B.	None ...	15	224	0	56	199	- 31	... 17.97	
	Soya milk	15	224	362	68	484	+ 34	... 24.9	
	Cow's milk	15	224	365	84	495	+ 60		

TABLE II.
Blood calcium (in mg. per c.c.).

Subject.	OBSERVATIONS AT THE END OF:		
	Basal period.	Fortified soya milk.	Cow's milk.
D. C. ...	10.0	10.8	10.8
P. C. ...	10.1	11.2	11.1
F. B. ...	7.6	9.5	10.5
D. B. ...	9.3	10.5	11.0
O. C. ...	9.4	9.8	10.0
L. R. ...	9.9	10.5	10.7

addition of about 363.5 mg. of calcium from either cow's milk or calcium-fortified soya milk changed the average daily calcium balance from -54.5 mg. to an average of +29.0 mg. in the case of soya milk and +28.9 mg. in the case of cow's milk. The average utilization of the supplementary calcium from soya milk for effecting this change of calcium balance was 23.1 per cent and that from cow's milk was 22.8 per cent. The average response to the two milks was practically identical.

Subjects F. B. and D. B. were constipated for a part of the time and subjects O. C. and L. R. had often unformed stools. Faecal calcium excretion, however, did not show much relationship to the frequency of defaecation. Thus, subject P. C. with normal defaecation excreted the most calcium in faeces and subject O. C. with frequent defaecation excreted less faecal calcium than subject P. C.

Specimens of blood sera were analysed at different stages of the experiment to determine the quantitative variations in the concentrations of calcium. In all the cases there was significant increase in calcium level following the addition of the supplement as either soya or cow's milk. The average increase in the case of the former was 1.2 mg. per 100 c.c. in the case of soya milk and 1.5 mg. per 100 c.c. in the case of cow's milk.

It is well known that normal persons receiving a well-balanced diet would show practically no variation in the calcium level of blood serum even if they receive supplements which are rich in calcium. In the present study, the basal diet was not adequate in regard to calcium, and as there was also a negative balance, there was naturally a striking response on supplementation with either soya or cow's milk. Such increase is also known following the injection or heavy oral administration of calcium in some concentrated form.

The percentage utilization of the supplementary calcium ranged from about 18 to 30 and averaged about 23 in the case of both fortified soya and cow's milk. Although there are individual variations, the averages are strikingly similar.

SUMMARY.

1. Comparative studies on the utilization of calcium from fortified soya milk and cow's milk were carried out with six growing children (age 7 to 9 years) who received the milks as supplements to a basal diet composed mainly of cereals and which provided only a small amount of calcium (224 mg.) per day.

2. When fed on the basal diet alone, the children showed a negative calcium balance, the excretion exceeding the intake by 20 mg. to 135 mg. per day. On supplementing the diet with fortified soya or cow's milk, the children received 362 mg. per day of calcium in the case of the former and 365 mg. per day from the latter. As the result of this, the calcium balance was changed from an average of -54 mg. per day in the case of the basal diet to +29.0 mg. in the case of soya milk and +28.9 mg. in the case of cow's milk.

3. In spite of the initial negative balance, the extent of utilization of the supplementary calcium was only 23.1 per cent in the case of soya milk and 22.8 per cent in the case of cow's milk. The response to the two milks was practically the same.

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SOYA-BEAN ASCORBICASE.

BY

Y. B. RANGNEKAR,

S. S. DE,

AND

V. SUBRAHMANYAN.

(From the Department of Biochemistry, Indian Institute of Science, Bangalore.)

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MUCH valuable work has been done on the isolation of ascorbic oxidase from leafy vegetables and tubers. Its existence in some fruits has also been observed (Tauber *et al.*, 1935; Hopkins and Morgann, 1936; Srinivasan, 1936; Johnson and Zilva, 1937). The preparation and behaviour of copper-protein complexes possessing similar properties have also been described. Divergent views exist, however, regarding the nature of the enzyme (Stotz *et al.*, 1937; Lovell-Jamison and Nelson, 1940; Ramasarma *et al.*, 1940; Lampitt *et al.*, 1944). No attempt seems to have been made to isolate and study the enzyme from germinating seeds, although its presence in germinating soya bean has been reported (Ito, 1938; Shen *et al.*, 1945). During the course of studies on vitamin C in soya bean and soya milk (Rangnekar *et al.*, 1948), it has been observed that the bean possesses a powerful ascorbicase activity which is dormant in the dry seed but develops quickly on germination. The important bearing which this aspect has on the final vitamin C values of the soya milk prepared from the bean after extended germination has also been discussed from the viewpoint of the scalding of bean prior to milk preparation. The present studies relate to the isolation, properties and chemical nature of soya-bean ascorbicase.

EXPERIMENTAL.

Determination of ascorbic oxidase activity.—Four grammes of the germinating bean were thoroughly ground with glass-distilled water in a glass-mortar and then centrifuged and made up to 20 c.c. To 10 c.c. of the centrifuged extract (pH about 6.0) were added 3 c.c. Sørensen's phosphate buffer pH 6.0 and 2 c.c. solution containing 2 mg. ascorbic acid, and the reaction mixture kept at 25°C. At regular intervals of time 2 c.c. of the solution were withdrawn, 20 per cent HPO₃ added to stop the reaction and the unreacted ascorbic acid titrated against standardized

2: 6 dichlorophenolindophenol. (Note.—In case of sprouts 0.2 g. were ground in 1 c.c. water and to it were added 0.2 mg. ascorbic acid in 1 c.c. phosphate buffer. The reaction was stopped after 20 minutes and the unreacted ascorbic acid then titrated.)

The results for the development of ascorbicae in germinating soya bean (in cotyledons as well as the sprouts) and of the activities as influenced by the varietal differences (Table I) show that the enzymic activity is developed practically at a

TABLE I-a.

Development of ascorbicae in germinating soya bean.

Period of germination in hours.	TIME OF REACTION IN MINUTES :			
	10	20	40	60
	Activity (as percentage ascorbic acid destroyed).			
0	0	0	0	0
24	13.3	30.6	41.2	64.6
48	31.5	32.5	64.8	100.0
72	29.2	47.4	67.5	88.3
96	26.3	47.6	59.8	79.9

TABLE I-b.

Results expressed in terms of percentage ascorbic acid oxidized in 20 minutes.

Period of germination in hours.	WHITE VARIETY.			Black variety.
	Cotyledons.	Sprouts.	Whole beans.	
	Activity (as percentage ascorbic acid destroyed in 20 minutes).			
0	0	0	0	0
24	21.3	66.4	30.6	24.5
48	40.7	89.2	52.5	46.4
72	41.6	78.7	47.4	44.4
98	40.1	58.7	47.6	45.2

maximum after 48-hour germination period and is more or less retained to that extent in cotyledons while decreasing in the sprouts. Another notable point is the higher production of the enzyme during germination of the white variety of the soya bean as compared to the black one. It may be recalled that the white variety produces vitamin C also to a greater extent than the black one (Rangnekar *et al.*, *loc. cit.*).

Preparation of ascorbicase from soya bean.—One kilo of 48 to 60 hours' germinated soya bean (white variety) was thoroughly macerated and ground in a stone-mortar, the juice was pressed out and the residue then extracted in small instalments with minimum amounts of glass-distilled water. After one or two such extractions the whole filtrate (about 500 c.c.) was treated with M Ba-acetate (5 c.c.) and was then left overnight in a cold-chamber when after long standing the protein matter was separated and centrifuged off along with excess of Ba-ion which was precipitated by addition of 5 c.c. saturated ammonium sulphate. The filtrate was collected and divided into three parts (A, B and C).

Part A.—Ammonium sulphate was added to saturate and the precipitate was separated by centrifuging. The crude enzyme was dissolved in minimum volume of $M_5KH_2PO_4$ (the resulting pH was 5.8) and centrifuged so as to remove other insoluble impurities. The enzyme was re-precipitated and re-dissolved as before and the process twice repeated again. The preparation (I) at this stage was muddy-brown in colour. One portion (II) was dialysed in a collodion bag against distilled water for 60 hours, while another (III) after dialysis was twice adsorbed on alumina at pH 6.4 and then eluted with secondary phosphate. This was followed by dialysis for 24 hours against glass-distilled water. All the three preparations were dried in a vacuum desiccator over concentrated H_2SO_4 . They were then powdered and used as such.

Part B.—Precipitated with 96 per cent alcohol, washed with 80 per cent alcohol and then dissolved in $\frac{M}{5}Na$ acetate (pH 6.0). The process was twice repeated and was followed by dialysis against distilled water for 60 hours.

Part C.—Precipitation done by acetone: This was not dialysed. Preparations from B and C were dried and compared with those from A.

Stability of the enzymic preparations.—The dried enzyme preparation (fraction I of part A) retained its full activity for $1\frac{1}{2}$ months when stored in an evacuated tube at $0^\circ C$. However, the suspension in phosphate buffer lost about 15 per cent activity during storage for the same period. The enzyme preparations from B and C proved to be unstable losing up to 50 per cent of their original activity. As would be seen later, there is inactivation of soya-bean ascorbicase by alcohol and acetone, especially the latter.

The activity of the enzyme preparations was determined by the method of Tauber *et al.* (*loc. cit.*) with a slight modification. The velocity of the reaction was studied at $37^\circ C$. and $M/15$ phosphate buffer of pH 5.9 to 6.0 was used. The reaction mixture consisting of 1 c.c. phosphate buffer, suitable quantity of the enzyme solution, ascorbic-acid solution (0.5 m.) and water to make final volume 5 c.c., was placed in 50 c.c. conical flasks and kept well shaken during the experiment. The quantity of the oxidase solution was so chosen that about half of the ascorbic acid was oxidized in 5 minutes. At regular intervals the unreacted

ascorbic acid was estimated by stopping the enzymic activity with 1 c.c. 20 per cent metaphosphoric acid and titrated against standard Tillman's reagent. The quantity of enzyme necessary for oxidizing 0.25 mg. of ascorbic acid in 5 minutes was calculated and this was taken as one unit of activity. The activity of each enzyme preparation was expressed quantitatively in number of units per g. of dry enzyme.

The copper content of the enzyme preparation was measured by the colorimetric method of Sylvester and Lampitt (1935).

Results for the activity of the different enzyme preparations, with successive degree of purification, and their Cü-contents in micrograms per g. are presented in Table II:—

TABLE II.
Cü-content and activities of soya-bean ascorbicase preparations.

Enzyme preparation.	Activity in units per g. of enzyme.	Cü-content in mg. per g. of enzyme.	Cü-content in mg. per unit equivalent of enzyme.
Crude enzymic extract	5
A—Ammon. sulphate precipitated—unpurified.	56	21	0.41
(i) Do. after repeated precipitation with ammon. sulphate.	584	86	0.15
(ii) A (i) after dialysis	873	101	0.11
(iii) A (ii) after twice adsorption on alumina.	1,147	118	0.10
B—(a) Alcohol precipitated, purified by re-precipitation.	148	69	0.47
(b) Do. dialysed	203	71	0.35
C—Acetone precipitated	94

It may be noted with successive purification the enzymic activity increases, so that the dialysed and adsorbed enzyme preparation (fraction III) is 230 times as much active as the crude enzyme extract. With increasing purification and greater activity of the enzyme preparation the Cü-content seems to increase. However, a strict relationship is not apparent. Although it could be said that Cü-content of the enzyme is a responsible factor for its activity, it is difficult to postulate that the entire enzymic activity is solely dependent thereon and that it is in direct proportion to the latter. For example, the difference in the Cü-contents of fractions A-II and III is not so significant, although purification through adsorption on alumina has considerably increased the activity from 873 to 1,147 units. Probably adsorption may have caused the removal of some factors inhibiting the activity, while at the same time not affecting the oxidase activity. Similarly, although dialysis has appreciably enhanced the activity in part B preparation, there is negligible change in the Cü-content. That dialysis is able to eliminate the inhibiting substances to a great extent may be seen from results in Table V.

The last column in Table II gives $\mu\text{g. of Cu}$ per unit equivalent of the enzyme, so as to facilitate the comparison of copper contents of the different enzyme preparations with increasing degree of activity. It would be seen that as purification approaches its maximum level (i.e., fractions A-II and III), the copper content per unit equivalent reaches nearly a steady value.

The de-activating influence of alcohol and acetone treatment on soya-bean ascorbicase is apparent from results in Table II for preparations B and C. Dialysis failed to bring up the activity to the level in preparation A (in contrast with results in Table V for inhibiting substances). Here again it is seen that although the Cu-content is nearing that in fraction (preparation A), the activity is less than half.

P_H MAXIMA OF SOYA-BEAN ASCORBICASE.

Generally the optimum pH for ascorbic oxidase has been denoted as 5.6 in phosphate buffer. Tauber *et al.* (*loc. cit.*) found this value for the preparation for Hubbard squash, while for the enzyme isolated from drumstick, Srinivasan (*loc. cit.*) reported it as 5.4 to 5.8. Many of the preparations from other sources have also given similar values. However, for soya-bean ascorbicase the optimum seems to be near 6.0—between the range 5.9 and 6.2 with Sørensen's phosphate buffer. In Table III are presented the activity of soya-bean ascorbicase at different pH, both in Sørensen's phosphate and in McIlvaine's phosphate-citrate buffer. It would be seen that with the latter the pH range is lower and the activity also less.

TABLE III.

Influence of pH on activity of soya-bean ascorbicase.

Reaction mixture 1 c.c. buffer + 0.5 mg. ascorbic acid + 1 c.c. enzyme, total volume 5 c.c.
Percentage original ascorbic acid oxidized in 5 minutes expressed as the activity. Temperature—37°C.

McILVAINE'S PHOSPHATE-CITRATE.		SØRENSEN'S PHOSPHATE.	
pH.	Activity.	pH.	Activity.
3.0	10.2	5.3	87.7
3.6	33.2	5.6	95.1
4.2	53.5	5.9	100.0
4.8	86.6	6.2	100.0
5.4 — 6.0	97.8	6.5	96.2
6.4	72.7	6.8	78.1
6.8	50.3	7.2	58.3
7.4	28.9	7.4	46.5
8.0	8.6	8.0	25.2

Importance of oxygen for enzyme action.—When 2 c.c. of the enzyme and 2 mg. of ascorbic acid in 2 c.c. phosphate buffer (pH 6.0) were kept in dark in evacuated Thunberg tubes in contact with 1 c.c. M/1,000 methylene blue, no perceptible discoloration of the dye was observed as compared in a visual colour comparator against two controls: first where air was not excluded and, second, where enzyme was substituted by water alone. This showed the essentiality of molecular oxygen for the oxidation.

SPECIFICITY OF SOYA-BEAN ASCORBICASE.

There was no action of the enzyme on catechol, pyrogallol, o- or p-cresol which suggested that the enzyme was not a polyphenol or indophenol oxidase. No action on these was observed in presence of H_2O_2 which indicated the absence of peroxidase.

The rate of oxidation of ascorbic acid by the enzyme was unaffected by addition of catechol, thereby indicating that the oxidation was brought about directly and not through the quinones produced from the polyphenols (*cf.* Johnson and Zilva, *loc. cit.*).

That the oxidation of ascorbic acid brought about by soya-bean ascorbicase is only up to the de-hydroascorbic acid stage—the reversibly oxidized form—was shown by the fact that when a solution containing 0.5 mg. ascorbic acid, completely oxidized by 1 c.c. of enzyme in 5 minutes (pH 6.0), was reduced with H_2S (overnight), the gas being removed next day with a current of N_2 , 0.489 mg. of ascorbic acid was recovered on titration with standard Tillman's reagent.

Soya-bean ascorbicase was unable to catalyse the oxidation of glucoreductones (produced by heating glucose with the alkali).

ENZYME KINETICS.

That the oxidation of ascorbic acid brought about by soya-bean ascorbicase is a reaction of first order was seen from the calculated values from Table I-a. The rate of oxidation was observed to be directly proportional to the enzyme concentration and was unaffected by increasing quantities of substrate.

The temperature optimum (Table IV) for the activity of soya-bean ascorbicase was found to be 37°C. There was little activity at 75°C., while at 85°C. it was practically inactivated.

TABLE IV.

Influence of temperature on activity of soya-bean ascorbicase.

(Conditions: similar to those in Table III.)

Temperature :—	25°C.	37°C.	50°C.	60°C.	75°C.	85°C.
Activity (percentage ascorbic acid destroyed).	91	100	74	35	10.1	2.3

Influence of inhibiting substances on the activity of soya-bean ascorbicase.

Results presented in Table V indicate that all the four Cu-inhibitors, KCN, Na-diethyl dithio carbamate, thiourea and H_2S , were very greatly inhibitive towards soya-bean ascorbicase also, KCN being the highest and thiourea the lowest. This would suggest the association of copper with the enzymes. At the same time the absence of complete inhibition is also significant.

TABLE V.

Inhibition of the activity of soya-bean ascorbicase.

Reaction mixture — 0.5 mg. ascorbic acid + 1 c.c. enzyme in a total volume of 5 c.c. in phosphate buffer (pH 6.0) at 37°C. One c.c. was pipetted out at time intervals and titrated. Results expressed as percentage ascorbic acid oxidized. Concentration of the inhibitors M/1,000.

Time in minutes.	Enzyme alone.	Enzyme + KCN.	Enzyme + diethyl dithio carbamate.	Enzyme + thiourea.	Enzyme H_2S .
1	31.1	5.2	7.4	9.8	4.1
3	78.4	9.8	10.6	17.1	9.2
5	100.0	12.0	14.7	21.5	12.6
Percentage inhibition.	...	88.0	85.3	78.5	87.4

From results given in Table VI, it would be observed that dialysis of the inactivated soya-bean ascorbicase solutions led to substantial recovery in activity. However, the regeneration is not complete and with the KCN inhibited enzyme the restoration is small. In the case of the enzyme extract from potatoes the activity of which was first inhibited by KCN, it has been reported by McCarthy *et al.* (1939) that the activity was completely restored on dialysis while in the case of cucumber extract it was not.

To test whether addition of Cu to inactivated soya-bean ascorbicase imparts its activity, consequent on formation of a complex with the protein, an experiment was conducted with KCN inactivated enzyme. The result is given in column 3 of Table VI. The effect seems to be comparatively little as against the practically complete oxidation due to Cu-ion above at that concentration. Obstruction in an effective formation of Cu-protein complex by the KCN may be possible. McCarthy *et al.* (*loc. cit.*) report a high degree of activity regeneration consequent on such an addition.

Five c.c. of the enzyme (phosphate buffer pH 6.0) inactivated with the copper inhibitors were dialysed against glass-distilled water for 24 hours. Its activity was then measured. It was also measured after addition of Cu at a concentration of 2×10^{-3} to the inactivated enzyme solution. This was compared with the oxidation of ascorbic acid (at above-mentioned conditions) with Cu alone at same

concentration. Results expressed as percentage of ascorbic acid in 5 minutes (conditions same as in Table V).

TABLE VI.

Regeneration of enzymic activity of soya-bean ascorbicase.

Inhibitors used.	Inhibited.	After dialysis.	After addition of $\text{Cu}-2 \times 10^{-3} \text{M.}$	Destruction of ascorbic acid only due to $\text{Cu}-2 \times 10^{-3} \text{M.}$
None	100
KCN M/1,000	12.0	18.1	25.3	98.2
Na-diethyl dithio carbamate	14.7	71.3
Thiourea	21.7	72.6

DISCUSSION.

Many points of interest emerge from the present investigation. A salient one is the association of copper with the enzyme. As mentioned earlier, this has been a subject of much controversy. The problem has been attacked from both sides, viz., synthetic copper-protein enzyme and natural ascorbic oxidase, so as to compare the properties of the two. McCarthy *et al.* (*loc. cit.*) from their works on synthetic Cu-gelatine and Cu-albumen mixtures, the properties of which they compared against natural ascorbic-oxidase preparations from cauliflower and squash, concluded that the enzyme is basically a copper-protein complex. However, some other workers have disputed the above conclusions. Thus, Graubard (1939) could not confirm the findings of McCarthy *et al.*, and he and other investigators like Straub (1938) and Sprugt and Vogelberg (1938) point out that the amount of copper present cannot completely account for the high catalytic activity of the plant juices and that the copper-protein complex does not act exactly like the enzyme. More or less similar views have been expressed by Lovell-Jamison and Nelson (*loc. cit.*) and Ramasarma *et al.* (*loc. cit.*). On the other hand, the recent findings of Lampitt *et al.* (*loc. cit.*) that the ascorbic-acid oxidizing properties of a mixture of $\text{Ca}(\text{HCO}_3)_2$, an alkali phosphate and a trace of ionized copper were destroyed by heating at 60°C. , seem to partly support the earlier views of McCarthy *et al.* Although the trend of the evidence obtained in the present investigations on soya-bean ascorbicase does point towards the view that copper is an essential part of the ascorbic oxidase and thus plays an effective rôle in its enzymic activity, it is difficult to say as to how far the association of the copper with the protein in the enzyme determines the activity. Thus even though with increasing purification and the resulting higher activity the copper content appears to increase, a strict relationship between the two is not evident. The copper-protein theory depends mainly on the constancy of relationship between

Cu and activity. Definite evidence to this effect is not indicated by the present studies. If the copper of this protein complex becomes partially ionized in aqueous solution, the catalytic activity would depend on the degree of ionization unless the protein fraction itself accelerates or retards the secondary reaction. In this connection, the finding during the present studies that Cu added externally to inactivated soya-bean ascorbicase did not appreciably restore its activity as compared with the practically complete oxidation of ascorbic acid due to Cu alone at that concentration, is of importance. However, the absence of complete inhibition is significant and this would suggest that all the activity is not due to the copper present. Further, the observation that the resting bean, although containing appreciable amount of copper, did not show any ascorbic-oxidase activity, while the germinated one did also point towards similar conclusion. All these considerations lead to the view that although copper is an active constituent of ascorbicase, it is not merely a copper-protein complex but has its own enzymic existence also.

It is interesting to note in the present studies that during germination of soya bean, the ascorbicase activity develops practically to maximum after 48-hour period, remaining more or less stationary after that period in the cotyledons while decreasing in the sprouts, although, however, the initial production of the enzyme is much higher in the sprouts than in the cotyledons. On the other hand, it has been observed that a greater percentage formation of vitamin C occurs in the cotyledon than in the sprouts in germinating seeds (Bhagvat and Rao, 1942). How far these two aspects are interrelated it is difficult to say at present. However, the significance of the observations would be evident when the changes in the formation of total reduced and de-hydroascorbic acid in the whole bean are taken into consideration (Rangnekar *et al.*, *loc. cit.*).

The difference in the optimum reaction of soya-bean ascorbicase (pH 6.0) as against that for the enzyme from other sources like cucumber (pH 5.6) is noteworthy. The association of some other factor with soya-bean ascorbicase may possibly be responsible for this apparent difference.

SUMMARY.

1. In germinating soya beans the ascorbicase activity is developed practically to a maximum after 48 hours. After that period, it remains more or less stationary in the cotyledons but decreases in the sprouts. There is higher production of the enzyme during germination in the white as compared with the black variety of the bean.

2. The isolation concentration and purification of the enzyme have been described and the activities of the different fractions indicated in terms of units per g. of the dry enzyme preparation. A detailed study of the Cu-content of the different fractions have been undertaken in relation to their activities and these show that Cu is an active constituent of ascorbicase. It is not, however, to be merely a Cu-protein complex but having its own enzymic existence.

3. Other properties of soya-bean ascorbicase have been studied. The optimum reaction is about 6.0 as compared with 5.6 for preparations from other sources.

4. Typical Cu-inhibitors have been observed to very greatly de-activate soya-bean ascorbicae. The inhibition is not, however, complete.

5. Dialysis of the de-activated preparations restores most of the original activity excepting in case of the KCN treated preparation. In the latter case, addition of copper produces only a slight regeneration in the activity. The restoration in case of KCN inhibitors is, however, small. Addition of Cu-ions to KCN de-activated enzyme regenerates the activity to some extent.

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STUDIES ON INDIAN EDIBLE OILS.

GROUND-NUT OIL.

BY

K. RAMAMURTI,

AND

B. N. BANERJEE.

(From the Department of Biochemistry, Indian Institute of Science, Bangalore.)

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THE nutritive value of a fat can be considered from several points of view. To serve as food, its assimilation and utilization in the metabolism are important. The first process, therefore, is digestion; while absorption comes later. Oils and fats are the carriers and solvents of vitamins and carotenes. The rôle of Indian edible oils in this aspect of the problem is vital for the health of the nation. The digestibility of the carotenoid foods and their retention is a great criterion in the tropics more so with the vegetarians of India. In a tropical country like India, oils and fats quickly go rancid, unless consumed, soon after their preparation. Therefore, this storage property is very important. In the cooking processes of frying, etc., the oil is exposed to high temperatures when oxidation and peroxide formation take place.

The Report of the Marketing Board on Ground-nut Oil (1941) gives a picture of the uncontrolled state of affairs and bad quality of the oil put on the market. The development of acidity on storage of nuts in Bombay and Madras has been recorded. The development of acidity has been found to be considerably increased by 'damaged', 'splits' and 'nooks'. Nothing is done by the crushers before extracting the oil; so that, oils with very high acidity are produced. No limit has been prescribed for the free fatty acidity of the oil that can be considered fit for edible purposes. Only hydrogenation factories refuse oils above a certain acidity and colour as it adds to their cost of manufacture and affects the yield. The Agmark has given no specifications for good quality edible oil. This is a deplorable state of affairs since high acid and rancid oils are known to destroy

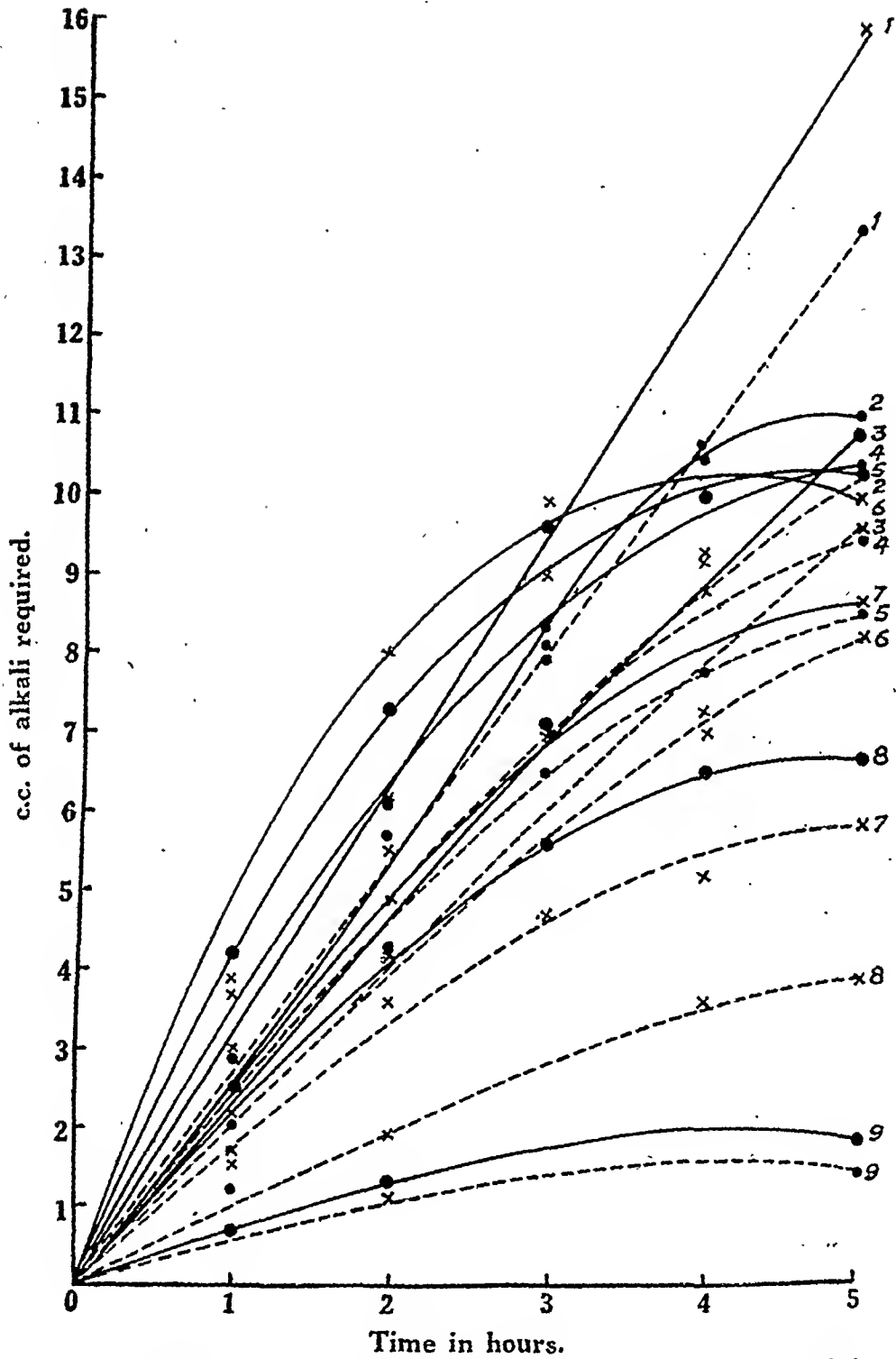


FIG. 1.—Showing rate of hydrolysis of high free fatty acidity ground-nut oil before and after frying.

(The dotted lines represent the fried samples.)

vitamins. It is, therefore, very important that a limit be prescribed for the acidity in edible ground-nut oil.

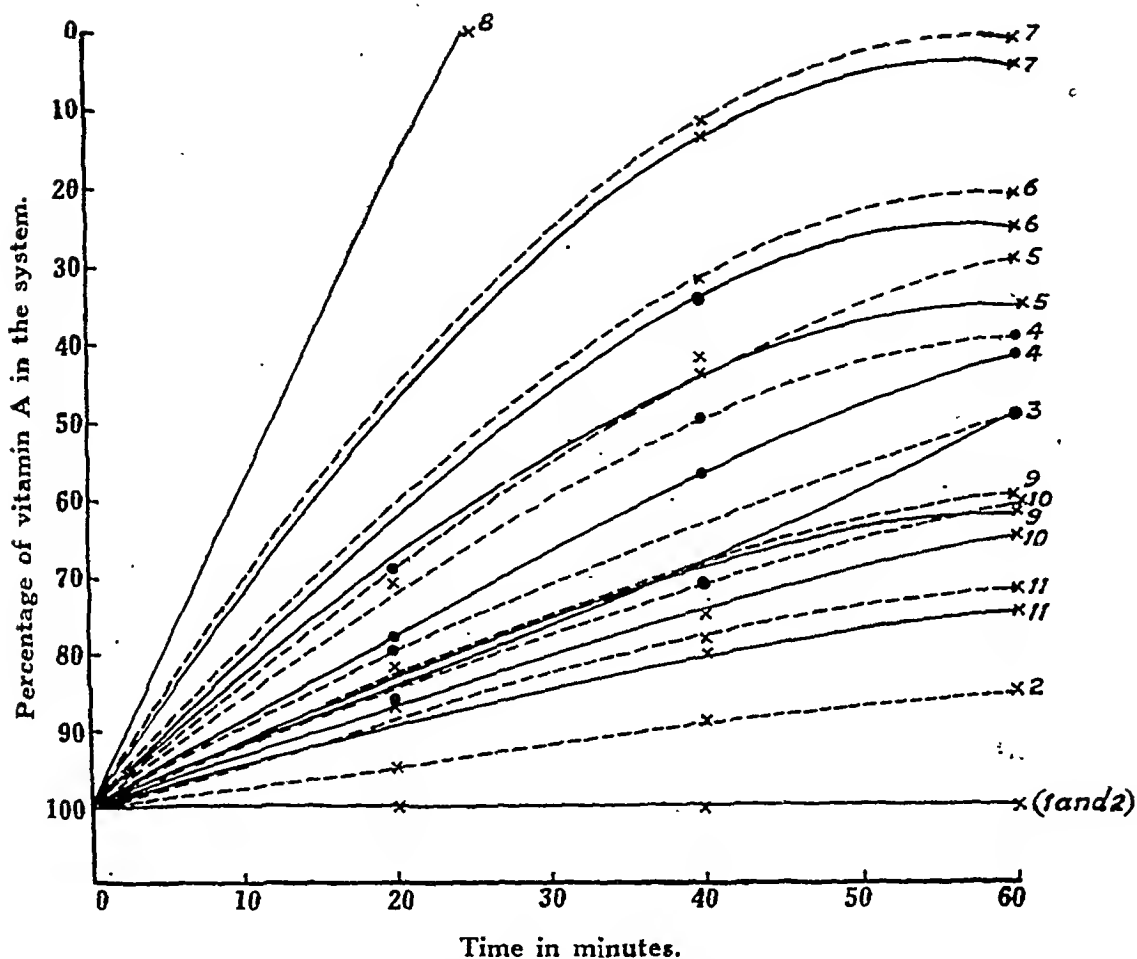


FIG. 2.—Loss of vitamin A in high acidity ground-nut oil before and after frying.
(The dotted lines represent fried samples.)

Samples of ground-nut oil obtained from Bangalore market have been studied for their (Part I) free fatty acidity (f.f.a.) and keeping quality; and (Part II) digestibility, effect on carotene and vitamin A, and storage with anti-oxidants. Since the trade in refined ground-nut oil is on the increase, the effect of various operations carried out during refining on each of the above aspects was also studied.

Part I.**FREE FATTY ACIDITY AND KEEPING QUALITY OF GROUND-NUT OIL.**

Twenty-four samples of ground-nut oil collected from all over Bangalore were analysed for their colour and free fatty acidity. The estimation of colour was undertaken to find out if any relationship existed between colour and acidity. Since estimation of colour is quicker than determination of acidity (if there was any direct relationship between colour and acidity) the limit for acidity could be easily translated into the limit for colour, thereby helping the lay public.

TABLE I.

Number.	COLOUR IN LOVIBOND UNITS.		Acidity as oleic acid, per cent.
	Yellow.	Red.	
1	1	...	1.1
2	6	1.1	1.1
3	8	1.0	2.0
4	5	1.1	2.4
5	5	1.0	2.4
6	6	1.0	2.5
7	5	1.1	2.6
8	3	0.2	2.6
9	5	0.1	2.6
10	6	1.0	2.8
11	5	0.9	3.0
12	7	1.0	3.1
13	6	1.3	3.1
14	7	0.8	3.3
15	7	1.4	3.3
16	6	1.0	3.3
17	7	1.1	3.3
18	9	1.1	3.5
19	9	0.2	3.6
20	5	0.1	3.8
21	6	1.1	4.2
22	5	0.9	4.5
23	8	1.4	5.6
24	10	1.9	7.7

It will be seen from Table I that the majority of market samples has acidity ranging between 2 and 4 per cent, while one sample has acidity as high as 7.6 per cent. Comparison of colour and acidity of the various samples reveals that the sample with the lowest acidity (1.1 per cent) is also the one with the lightest colour and the sample with the highest acidity is also the one with the deepest colour. However, in the case of many samples, colour and acidity do not run parallel to one another. It is unsafe to relate colour and acidity directly, though in several cases a dark colour may also mean a greater acidity. A very pale straw-yellow colour can be considered satisfactory.

Most of the shops receive the oil from the oil mills while a few get from the village 'teli'. It was, therefore, considered advisable to analyse a few fresh samples from the oil mills and from the *ghani* (indigenous oil-presser) to know if the cause of the high acidity and deep colour of the market samples was at the source itself. The relative keeping quality of expeller and *ghani* samples was also ascertained by determining the induction period, which is the phase of very slow change which proceeds rapid oxidation of the fat. The method of Holm and Greenbank (1925) was used for the determination of the induction period.

TABLE II.

Oil.				COLOUR IN LOVIBOND UNITS.		Acidity as oleic acid, per cent.	Induction period in hours at 60°C.
				Yellow.	Red.		
1.	Expeller	5.2	...	2.2	2½
2.				8.0	1.0	2.03	2
3.				1.0	...	1.1	2
4.	Ghani	4.0	...	1.1	3
5.				5.1	1.1	2.38	1½

While the acidity of fresh samples obtained directly from the oil mills and the *ghani* is not so high as that of the market samples, it is nevertheless sufficiently high. Inquiry at the oil mills and the village 'teli' showed that no grading or selection of the seeds was made before expelling the oil, i.e., both good and bad ones were used together.

Experiments were undertaken to find out how far the absence of such a selection was responsible for the increased acidity of the oil. The following procedure was adopted:—

Shelled ground-nut seeds stored in gunny bags were obtained from the market. The sample thus obtained contained both good and damaged seeds. This was divided into four batches. The first and the second batches consisted of healthy hand-picked seeds, the third of shrivelled and broken grains, and the fourth of mouldy grains. The seeds of the first batch were cold-pressed, those of the second batch were heated in steam and then pressed for oil, while the third

and fourth batch seeds were both cold-pressed. The colour, acidity and induction period of the different samples are given in Table III :—

TABLE III.

Batch.	Colour in Lovibond units (yellow).	Acidity as oleic acid, per cent.	Induction period in hours at 60°C.
I	2.0	0.6	8
II	5.0	1.1	5
III	2.0	4.5	2½
IV	2.5	3.5	3

Table III shows that when healthy hand-picked seeds alone are chosen, the acidity of the oil is very much lower than 1 per cent (only 0.6 per cent) when cold-pressed and only slightly more than 1 per cent (1.1 per cent) when hot-pressed, whereas mouldy, shrivelled and broken seeds yield an oil of acidity between 3.5 and 4.5 per cent when cold-pressed. The keeping quality of the oil as shown by the induction period diminishes from batch I to batch IV. Thus, it is seen that damaged seeds play a considerable rôle in increasing the acidity and lowering the induction period of the oil, whether it be obtained from the expeller or from the *ghani*.

The very high acidity of market samples, as revealed in Table I, cannot, however, be completely explained on the above basis. The unsatisfactory conditions of storage obtained in shops must also materially contribute to the deterioration of the oil.

EFFECT OF REFINING ON THE QUALITY OF THE OIL.

One expeller sample, one *ghani* sample and all the four laboratory samples were refined—first by treatment with caustic soda (at 60°C. for one hour), then by decolorization with kaolin and animal charcoal (at 60°C. for one hour) and finally deodorized by passing steam for one hour. Table IV gives the colour, acidity and induction period of the samples after each stage in refining process.

A careful scrutiny of Table IV shows that, whereas samples of different batches show widely varying induction periods, members of the same batch show practically the same induction period after each stage in the refining operation. Thus, the difference in the induction periods of crude and refined samples of the same batch is very little, thereby showing that operations carried out during refining do not improve the keeping quality of the oil to any considerable extent. After refining a crude oil one should naturally expect that since the refined product has a very low acidity the latter should keep better. Table IV shows that this is not so. While refining may lower the acidity and make the oil more agreeable to taste and odour, its effect is but little in so far as the keeping quality of the oil is concerned. The harm done, as expressed by the initial acidity of the crude

TABLE IV.

Nature of the sample.	COLOUR IN LOVBOND YELLOW UNITS.				ACIDITY AS OLEIC ACID, PER CENT.				INDUCTION PERIOD IN HOURS AT 60°C.			
	a	b	c	d	a	b	c	d	a	b	c	d
I	2.0	1.1	1.0	0.5	0.6	0.05	0.05	0.05	8	8½	9	10
II	5.0	3.9	3.0	0.6	1.1	0.17	0.2	0.09	4½	5	5½	4½
III	2.0	0.5	0.4	0.2	4.5	0.2	0.2	0.3	2½	2½	2½	3
IV	2.5	1.0	0.6	0.3	3.5	0.13	0.15	0.14	3½	3	3	3½
Expeller oil ...	5.2	2.5	2.0	1.5	2.2	0.33	0.27	0.28	3½	2	2	1½
Ghani oil ...	4.0	2.7	2.0	1.2	1.1	0.25	0.24	0.25	3	2½	2½	2½

a = crude oil ; *b*, *c*, *d* and *e* represent the oil after treatment with alkali, kaolin, animal charcoal and steam, respectively.

sample, persists on the keeping quality of the oil even after refining. No wonder, then, that anti-oxidants should be widely used to prevent rancidity in the finished products. Some hydrogenated products are being protected with anti-oxidants.

SUMMARY.

1. There is no direct relationship between colour and acidity of ground-nut oil, though broadly they are similar.
2. The incorporation of broken, mouldy and shrivelled grains raises the acidity 3 to 4 times.
3. Subjecting the seeds to heat-treatment before extraction of the oil nearly doubles the acidity.
4. The process of refining a high f.f.a. oil does not help in increasing its keeping quality.

Part II.

A. DIGESTIBILITY OF HIGH-ACIDITY GROUND-NUT OIL AS MEASURED BY ITS RATE OF HYDROLYSIS BY PANCREATIC LIPASE (*in vitro*).

Market samples of ground-nut oil with acidity ranging from 1 to 7 per cent were used in the experiment. Fresh ground-nut oil of very low acidity prepared from healthy hand-picked seeds in the laboratory was used as control. Fresh cow ghee, being the most easily digestible fat, was used for comparison. The hydrolysis was carried out with swine lipase prepared according to the procedure of Willstatter and Waldschmidt-Leitz (1923).

The hydrolysis was carried out using the experimental procedure of Weinstein and Wynne (1935-36) with a slight modification. A series of conical flasks (each of 50 c.c. capacity) was set up, each containing 2 g. of fat (emulsified with egg-albumin and gum acacia) and 1 c.c. of the buffer (ammonia-ammonium chloride buffer of pH 8.9) was used. One c.c. of the enzymic extract was added and immediately shaken with hand for three minutes and kept at a temperature of 37°C. The reaction mixture was removed at an interval of every one hour and 20 c.c. of 5:1 alcohol-ether mixture added and titrated against N/10 alkali using phenolphthalein as indicator. The reagents required for the experiment were prepared according to the method of Weinstein and Wynne (*loc. cit.*). The titration value of control mixture containing boiled enzyme, but otherwise identical with the active digest, was taken to represent the zero point.

The experiments were carried out both with raw as well as cooked (by frying chips) oil: The frying operation was carried out as is done in practice. The frying-pan was initially heated and measured volume of the oil poured into it. A known amount of the material to be fried was then added and the temperature noted. The time required for the completion of the frying as well as the maximum temperature attained were, as far as possible, the same in all cases.

The values given in Table V represent the average of triplicate determinations:—

TABLE V.

Sample.	Initial acidity of fat, per cent.	C.C. OF N/10 ALKALI REQUIRED AT REGULAR TIME INTERVALS.									
		1 hour.		2 hours.		3 hours.		4 hours.		5 hours.	
		Raw.	Fried.	Raw.	Fried.	Raw.	Fried.	Raw.	Fried.	Raw.	Fried.
1. Fresh cow ghee	0.1	3.0	2.8	6.2	5.7	9.0	8.3	12.3	10.6	16.0	13.4
2. Fresh ground-nut oil	0.6	2.0	1.7	4.4	3.6	8.1	7.9	10.5	8.8	11.0	10.2
3. {	1.1	2.5	2.2	5.8	4.2	9.6	9.0	10.0	9.3	10.8	9.6
4. {	2.0	3.6	2.9	8.0	6.2	8.0	7.1	9.2	7.8	10.4	9.5
5. {	3.3	4.2	2.6	7.3	5.4	10.1	6.4	10.8	7.2	10.3	8.2
6. {	4.2	6.2	2.1	9.5	4.4	9.9	7.0	10.1	7.8	10.0	8.3
7. {	5.6	3.9	2.4	4.9	4.2	7.0	4.7	7.0	5.2	8.6	6.9
8. {	7.7	2.9	1.6	4.8	1.9	5.6	2.2	6.5	3.6	6.6	3.9
9. Highly rancid	20.0	1.2	0.7	1.3	1.1	1.6	1.1	1.8	1.1	1.8	1.4

DISCUSSION.

Fresh cow ghee is the most easily hydrolysed fat and has been taken as the standard for comparison. Fresh ground-nut oil shows a satisfactory hydrolysis and up to samples possessing 2 per cent acidity there is a slight and gradual fall in the rate of hydrolysis. With higher acidities the fall is very rapid; and sample 9, a highly rancid sample, is hydrolysed only very little. There is a gradual decrease in the rate of hydrolysis of the oil, with increasing acidity of the oil. This applies both to the raw as well as fried oil.

The fall in the rate of hydrolysis after cooking the oil is more marked in the case of samples possessing more than 2 per cent f.f.a. An examination of the hydrolytic curve with pancreatic lipase shows that an acidity above 1 per cent is undesirable. It is possible to prepare such an oil if fresh healthy nuts alone are used for crushing, and 'damaged', 'splits', 'broken' and 'nooks' are carefully discarded. While a certain amount of damage is bound to occur in nuts during storage and transport, they should be separated from healthy nuts before crushing. The percentage of damaged nuts is not more than 10 per cent. The Marketing Board Report (*loc. cit.*) bears out this point. High acid oils obtained from non-graded nuts can be well utilized for preparing non-edible products for which there is ample demand. It is essential that the oil for edible purposes should be pressed from selected seeds and that considerations of trade should not be allowed to influence the quality of oil obtained for edible purposes.

B. EFFECT OF HIGH F.F.A. GROUND-NUT OIL ON VITAMINS AND CAROTENE.

Pure cow ghee of very low acidity (0.1 per cent) was used as the source of carotene. Since the sample of cow ghee gave a poor colour with antimony trichloride reagent for vitamin A, fresh shark-liver oil of low acidity was used as source of vitamin A.

When ground-nut oils of different acidities were mixed with ghee, the reaction was very slow to be measured in the ordinary way. Therefore, Sumner and Sumner's (1940) method was followed with a little modification. They have shown that fat-free soya-bean meal contains an enzyme carotene oxidase which oxidizes carotene, the reaction being catalysed by oils and fats and depending on the nature of the fat. Without a lipid catalyst, the reaction is very slow.

EXPERIMENTAL PROCEDURE.

(i) *Preparation of the enzyme solution.*—Five grammes of fat-free soya-bean meal was stirred with 200 c.c. of distilled water and the suspension was centrifuged for half an hour. The somewhat milky supernatant liquid was poured off and kept in an ice-chest.

For each sample of oil four sets of experiments were set up, the first one for recording the carotene content at the start, and the other three for finding out the carotene content every half an hour after the commencement of the experiment. A blank experiment, identical with others in all respects but without the enzyme, and a control experiment containing all ingredients except ground-nut oil, were also set up for purposes of comparison.

The experiment was carried out as follows: The ghee was melted till it was clear. Two c.c. of the ghee and 1 c.c. of ground-nut oil were pipetted out into a glass-stoppered conical flask containing 3 c.c. of petroleum ether and 2 c.c. of phosphate buffer of pH 6.5. Three c.c. of the enzymic extract were then added and the reaction mixture diluted with 10 c.c. of distilled water. The flask was kept in a thermostat at 37°C. with constant shaking. The carotene in the petroleum ether layer was estimated every half an hour, both by Klett colorimeter and the Lovibond tintometer. The estimations were repeated a number of times till complete agreement was reached by both methods. The results are expressed in number of mg. of carotene per 100 g. of fat in Table VI :—

TABLE VI.

Loss of carotene with high-acidity ground-nut oil.

Carotene content of ghee used 0.24 mg. per 100 g.

Sample.	Acidity as oleic acid, per cent.	Mg. of carotene per 100 g. of ghee at regular intervals of half an hour.		
		$\frac{1}{2}$	1	1½
1. Blank	0.24	0.24	0.24
2. Control	0.24	0.24	0.24
3. Fresh ground-nut oil ...	0.4	0.24	0.24	0.24
4. } Market sample ... {	1.4	0.24	0.21	0.22
5. }	2.3	0.21	0.17	0.17
6. }	3.4	0.19	0.16	0.14
7. }	4.2	0.15	0.09	0.06
8. }	5.3	0.13	0.07	0.05
9. }	10.4	0.06	0.04	0.01
10. Refined oil from crude oil of 2 per cent f.f.a.	0.1	0.23	0.21	0.19
11. Refined oil from crude oil of 1 per cent f.f.a.	0.15	0.24	0.23	0.20
12. Hydrogenated product from crude oil of 1.8 per cent f.f.a.	0.05	0.23	0.20	0.21
13. V.O.P. obtained from crude oil of 1.6 per cent f.f.a.	0.1	0.23	0.21	0.19

Loss of vitamin A (in vitro) with high-acidity ground-nut oil.

The effect of the following samples of ground-nut oil on vitamin A have been studied :—

- (i) Crude ground-nut oil of high f.f.a. before and after frying.
- (ii) Refined and hydrogenated ground-nut oil obtained from crude oils with high f.f.a.
- (iii) Crude ground-nut oil treated with anti-oxidant.

Progallin A (ethyl gallate) was used at a concentration of 0.03 per cent.

EXPERIMENT.

Two c.c. of fresh shark-liver oil was dissolved in 10 c.c. of petroleum ether.

One c.c. of ground-nut oil was dissolved in 2 c.c. of petroleum ether and 1 c.c. of the shark-liver oil solution was added and the mixture thoroughly shaken and incubated at 37°C. Of the reaction mixture 0.2 c.c. was pipetted out every 20 minutes and the vitamin A estimated by the Carr-Price method. The results are shown in Tables VII and VIII :—

TABLE VII.

Loss of vitamin A in ground-nut oil.

Sample.	Acidity as oleic acid, per cent.	PERCENTAGE OF VITAMIN A AT REGULAR INTERVALS.					
		20		40		60	
		Raw.	Cooked.	Raw.	Cooked.	Raw.	Cooked.
1. Blank	100	100	100	100	100	100
2. Fresh ground-nut oil ...	0.4	100	95	100	89	100	85
3. } Market sample ...	1.4	85.7	80	71.4	70	50	50
4. }	2.3	85.7	78	57.2	50	42.8	40
5. }	3.4	71.4	69	45.7	42	35.7	30
6. }	4.2	57.1	55	35.7	32	25.7	22
7. }	5.3	35.7	30	14.3	12	5	2
8. }	10.4	Nil	Nil	Nil	Nil	Nil	Nil.
9. Refined oil from crude oil of 1.6 per cent f.f.a.	0.15	85	82	75	75	62	60
10. Refined oil from crude oil of 2 per cent f.f.a.	0.10	86	84	78	75	65	61
11. Hydrogenated product from crude oil of 1.6 per cent f.f.a.	0.20	84	82	80	78	75	72

TABLE VIII.

Effect of ground-nut oil treated with progallin A on the loss of vitamin A.

Acidity of the oil, per cent.	TIME TAKEN FOR THE LOSS OF 20 PER CENT VITAMIN A.	
	Control.	Control and progallin A.
0.6	2 hrs.	5 hrs. 45 min.
1.4	1 hr.	3 hrs.
2.3	40 min.	1 hr. 20 min.
3.4	20 "	25 "
4.2	12 "	12 "
6.2	8 "	8 "

DISCUSSION.

Both carotene and vitamin A are inactivated (*in vitro*) in the presence of high f.f.a. ground-nut oil. The inactivation of vitamin A is greater when the oil is fried than when it is raw. Refining and hydrogenation of a high-acidity ground-nut oil do not prevent the inactivation to any considerable extent. The addition of an anti-oxidant like progallin A (ethyl gallate) is effective in prolonging the period of inactivation of vitamin A only so long as the f.f.a. of the oil is below a certain limit. As the f.f.a. of the oil increases the protection afforded by the anti-oxidant to vitamin A decreases. An oil of less than 1 per cent f.f.a. causes very little inactivation of carotene or vitamin A.

C. STORAGE OF GROUND-NUT OIL USING ANTI-OXIDANTS.

In order to study the storage property of the oils treated with anti-oxidants, the induction period of the samples were determined at 45°C. by the oxygen absorption method of Holm and Greenbank (*loc. cit.*) modified by Govindarajan and Banerjee (1939). Well-known anti-oxidants—propyl gallate, ethyl gallate (progallin P and progallin A of Nipa Laboratories, London), and hydroquinone were tried. The anti-oxidants were triturated with the oil and used at a concentration of 0.03 per cent.

The following samples of ground-nut oil were used: (i) from hand-picked healthy seeds, (ii) mouldy seeds, (iii) broken and shrivelled seeds. Sample (iv) was the same as (i) but the oil was pressed after steaming the nuts. Samples (i) to (iv) belong to the same batch of nuts. Samples (vi) and (vii) represent

market samples, while sample (v) is refined oil obtained from (vi). The results are given in Table IX:—

TABLE IX.

Storage property of ground-nut oil treated with anti-oxidants.

Number.	Acidity as oleic acid, per cent.	INDUCTION PERIOD AT 45°C. IN HOURS.			
		Control.	Control plus progallin P.	Control plus progallin A.	Control plus hydroquinone.
1	0.8	7	29	32	19
2	4.2	3	6	8	4½
3	5.9	2	4	5	2
4	1.6	6	20	22½	13
5	0.3	5	16	19	12
6	2.2	5½	15	17	13
7	3.5	4	9	10	6

DISCUSSION.

Of the three anti-oxidants tested, progallin A is the best for ground-nut oil. The protection afforded is 2 to 4 times more than the untreated oil. With increasing f.f.a. in the oil the storage property is poorer and the use of an anti-oxidant then fails to protect the oil in the same ratio. The removal of f.f.a. from a high-acidity oil does not improve the storage property and even the addition of an anti-oxidant then fails to increase its storage life. Therefore, from the point of rancidity, in the case of oils with acidity over 1 per cent, the storage quality is proportionally reduced and is improved only very little on refining and on the addition of an anti-oxidant. Above 4 per cent f.f.a. the storage life is *nil*, i.e., the oil is developing rancidity autocatalytically which cannot be checked.

SUMMARY.

From the point of view of digestibility, storage property, and as carrier and solvent for carotene and vitamin A, ground-nut oil below 1 per cent f.f.a. alone should be considered suitable for edible purposes. Removal of acidity or refining a high f.f.a. oil fails to bring it to the quality of fresh low f.f.a. oil. Any oil of over 2 per cent acidity should be rejected for edible purposes. Gallates, especially progallin A (ethyl gallate), increase the storage property 2 to 4 times.

Our thanks are due to Professor V. Subrahmanyam for his kind interest in the work.

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SYNERGISM IN CHEMOTHERAPY.

Part II.

FURTHER STUDIES ON PENICILLIN-DYE SYNERGY UPON GRAM-NEGATIVE BACTERIA.

BY

(Miss) MARIAM GEORGE,

AND

K. M. PANDALAI.

(From the Department of Biochemistry, Indian Institute of Science, Bangalore.)

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THOUGH the general belief that most Gram-negative bacilli are relatively resistant to penicillin has led to undue pessimism with regard to its therapeutic use in Gram-negative bacterial infections, many workers have explored the possibility of many Gram-negative bacilli becoming sensitive to higher concentrations of this substance (Helmholtz and Sung, 1944; Schwartzman, 1946). Thomas and Levine (1945) showed that certain intestinal bacilli were inhibited by 5 to 50 units and that involution forms appeared in sub-inhibitory concentrations. Stewart (1945) in his studies showed that some strains of *Proteus* could be inhibited in broth and body-fluids by 5 to 25 units/c.c. Since these higher concentrations are not maintainable under clinical conditions, it was regarded as worth while to try the combinations of penicillin with some other bacteriostatic agents in the hope of producing a synergic action whereby certain strains could be rendered vulnerable to lower concentrations of penicillin.

The conception of a synergic action upon certain organisms by penicillin and sulphonamide is a familiar one and it is becoming increasingly recognized. Ungar (1943), Bigger (1944) and Hobby and Dawson (1944) showed that the synergic action was essentially a bacteriostatic one. Bigger (1946) and Klien and Kalter (1946) contended that the synergic action was possible only when both agents were present in concentrations which were already inhibitory. Previous studies in this Laboratory (George and Pandalai, 1946), using *Staph. aureus* and *Esch. coli* as test organisms, showed that there was definite synergic action between penicillin and bacteriostatic dyes like gentian violet, methylene blue and brilliant green when the two drugs were used in sub-minimal bacteriostatic concentrations.

Based on the above suggestions, it was planned to investigate this synergic action against more Gram-negative organisms by using a number of bacteriostatic dyes combined with penicillin.

METHODS.

Media.—Nutrient broth of pH 7.0 was tubed in required volume so that after adding the requisite amounts of penicillin and dye, the final volume came to 10 c.c.

Penicillin.—Sodium penicillin (Lilly), with a potency of 100,000 units, was used.

Dyes.—The samples of basic dyes used in this study were all extra-pure type and specially manufactured for use as staining agents. The dyes studied were: (1) gentian violet, (2) methylene blue, (3) brilliant green, (4) basic fuchsin, (7) safranin and (8) acriflavine.

Inoculum.—The organisms were subcultured for 3 days in fresh nutrient-broth tubes and a 3-mm. loopful of 16-hour old broth culture was used throughout. A suitable dilution was used for colony counts in agar to give the approximate number of organisms in the inoculum and in the growing cultures. The test organisms were: (1) *B. dysenteriae* (Shiga) and (2) *B. typhosus*.

Readings.—The inhibitory effect of the penicillin and the dyes individually as well as combined was estimated by visual turbidity according to the standard shown in Table I:—

TABLE I.

Visual turbidity standard for estimating inhibitory effect of penicillin and dyes.

Degree of turbidity.	Appearance.
—	Clear.
±	Slight opalescence or granular deposit.
++	Turbid or granular.
+++	Turbid.
++++	Full 24-hour turbidity of control culture.

This technique has been used by previous workers (Bigger, *loc. cit.*; Stewart, *loc. cit.*) and found reliable and satisfactory. The method of visible turbidity observations has the added advantage that there cannot be any source of error since in all cases one can note the minimum inhibiting or bacteriostatic concentrations (m.i.c. or m.b.c.) of each drug in each experiment.

Tubes were inoculated and incubated at 37°C. and readings were taken at 24 hours and 48 hours and often even after 72 hours. A clear tube at the end of 72 hours was interpreted as complete lysis of the inoculum.

The first experiments done were designed to determine the minimum amount of penicillin and the dye solutions required individually to inhibit the growth of the test organisms in plain broth. A second series was done to study the

TABLE II.
Titration of penicillin and dyes individually against B. dysenteriae (Shiga).

Penicillin units/c.c.	CONCENTRATION OF DYES:					
	Methylene blue.	Brilliant green.	Gentian violet.	Basic fuchsin.	Safranine.	Acridflavine.
20.0	1/10,000	—	1/10,000	1/10,000	—	1/10,000
18.0	1/20,000	—	1/50,000	1/20,000	±	1/50,000
15.0	1/30,000	—	1/100,000	1/30,000	+	1/100,000
12.0	1/50,000	—	1/200,000	1/40,000	++	1/200,000
10.0	1/100,000	+	1/300,000	1/50,000	++	1/300,000
5.0	1/200,000	++	1/500,000	1/100,000	+++	1/500,000

combined action of sub-minimal amounts of penicillin and the dyes against the test organisms.

This organism is rather too resistant to penicillin as it needs 20 units per c.c. and upwards for complete inhibition. Brilliant green seems to be the best bacteriostatic agent in this case and then comes gentian violet and then acriflavine.

TABLE III.

Titration of penicillin with or without dyes against B. dysenteriae (Shiga).

Penicillin alone, units/c.c.	WITH DYES IN BROTH:					
	Methylene blue.	Brilliant green.	Gentian violet.	Basic fuchsin.	Safranine.	Acriflavine.
	1/20,000	1/200,000	1/200,000	1/40,000	1/20,000	1/200,000
15.0 +	—	+	—	—	—	±
12.0 +	—	+	—	—	±	±
10.0 +	±	+	—	±	±	±
8.0 ++	±	+	±	+	±	±
5.0 ++	±	—	±	±	±	±
0.0 +++	±	±	±	±	±	±

No synergism was manifest with brilliant green and acriflavine even though the dyes alone were strongly bacteriostatic. Gentian violet is found to be very active in bringing about the synergism.

TABLE IV.

Effect of varying amounts of penicillin and dyes on B. dysenteriae (Shiga).

Penicillin alone, units/c.c.	CONCENTRATION OF DYES:								
	Methylene blue.			Gentian violet.			Basic fuchsin.		
	1/20,000	1/30,000	1/40,000	1/200,000	1/300,000	1/400,000	1/40,000	1/50,000	1/60,000
15.0	—	—	+	—	—	—	—	±	+
14.0	—	—	+	—	—	±	—	±	+
12.0	—	—	+	—	—	—	—	+	+
10.0	±	+	+	—	—	+	+	+	+
8.0	±	+	++	±	+	++	++	++	++

TABLE V.
Titration of penicillin and dyes individually against B. typhosus.

Penicillin, units/c.c.	Dyes:					
	Methylene blue.	Brilliant green.	Gentian violet.	Basic fuchsin.	Safranine.	Acridflavine.
20.0	+	1/10,000	—	1/10,000	+	1/10,000
18.0
15.0	+	1/20,000	—	1/20,000	++	1/20,000
12.0	+	1/50,000	—	1/50,000	++	1/50,000
10.0
0.0	++	1/100,000	+	1/100,000	++	1/100,000

Methylene blue and safranine have no bacteriostatic activity against the test organism. Brilliant green and gentian violet are found to be good bacteriostatic agents. *B. typhosus* is less resistant to penicillin than *B. dysenteriae*.

TABLE VI.

Titration of penicillin with or without dyes against B. typhosus.

Penicillin alone, units/c.c.	WITH DYES IN BROTH:					
	Methylene blue.	Brilliant green.	Gentian violet.	Basic fuchsin.	Safranine.	Acridlavine.
	1/10,000	1/750,000	1/100,000	1/20,000	1/10,000	1/50,000
15.0	+	+	—	—	+	—
14.0	+	+	—	—	+	—
12.0	+	+	—	—	+	—
10.0	++	+	—	—	++	—
5.0	++	+	+	±	++	—
3.0	+++	+	+	+	+++	+

Methylene blue and safranine alone or with penicillin have no activity. Though brilliant green alone was bacteriostatic, there is no synergism with penicillin.

TABLE VII.

Effect of varying amounts of penicillin and dyes on B. typhosus.

Penicillin alone, units/c.c.	CONCENTRATION OF DYES:								
	Gentian violet.			Basic fuchsin.			Acridlavine.		
	1/100,000	1/125,000	1/150,000	1/20,000	1/30,000	1/40,000	1/50,000	1/60,000	1/75,000
15.0	—	—	+	—	—	+	—	—	+
14.0	—	—	+	—	+	+	—	—	+
12.0	—	—	+	—	+	+	—	+	+
10.0	—	±	+	—	+	+	—	+	+
8.0	—	+	+	—	+	+	—	+	+
5.0	—	+	+	+	+	+	+	+	+
3.0	±	+	+	+	+	+	+	+	++
2.0	+	+	+	+	+	++	+	+	++

Though penicillin as such has no inhibiting power up to 15 units per c.c., with a non-bacteriostatic concentration of gentian violet and acridlavine even 5 units per c.c. inhibited the growth.

GENERAL FINDINGS.

Penicillin.—Even though both the test organisms were Gram-negative intestinal bacilli, the sensitivity was slightly different. *B. dysenteriae* seems to be more

resistant to penicillin than *B. typhosus*. *B. dysenteriae* required 12 to 20 units per c.c., while *B. typhosus* was inhibited under the same conditions by 10 to 18 units per c.c. The concentrations shown above refer to 24-hour turbidity readings. When earlier readings were taken, suppression of growth was found to occur at lower concentrations during the first 5 to 8 hours at which stage the control cultures produced markable turbidity.

Dyes.—Sensitivity varied considerably in this case also. Methylene blue and safranin show very slight or no bacteriostatic activity against both the test organisms. Brilliant green and gentian violet are found to be the most active in comparably higher dilutions.

Synergic action.—It will be observed that, in general, the results of these studies indicate that there are synergic effects and that there is also some sort of uniformity in the results. However, it has to be pointed out that in the case of *B. dysenteriae* there is no synergic effect with penicillin and brilliant green, and penicillin and acriflavine. Gentian violet with penicillin on the other hand yields a good synergic effect. It may be recorded that basic fuchsin, methylene blue and safranin have very slight activity when present along with penicillin.

In the case of typhoid organisms (see results in Tables V, VI and VII) methylene blue and safranin alone or in the presence of penicillin show no bacteriostatic activity. Brilliant green up to 1/750,000 is bacteriostatic but no synergism with penicillin is obtained. The other dyes manifest synergistic effects and gentian violet is prominent in this aspect just as it was observed in the case of *B. dysenteriae*. In the case of basic fuchsin the penicillin concentration has gone down to 9 units per c.c. These results revealed the interesting point that there is evidence of very marked synergic effects when subminimal doses of the two drugs are used.

Size of inoculum.—The standard inoculum was one loopful of 3-mm. diameter (3×10^6 and 4×10^5 respectively) and the results shown in Table VIII refer to this.

TABLE VIII.

Effect of the respective synergistic concentrations of penicillin and dyes on varying numbers of the respective organisms.

Organisms.	Number of organisms, loops.	SYNERGIC CONCENTRATIONS OF PENICILLIN AND DYES :			
		Methylene blue.	Gentian violet.	Basic fuchsin.	Acriflavine.
<i>B. dysenteriae</i> (Shiga) ... {	1	—	—	—	} No synergism.
	2	—	—	—	
	3	+	+	+	
<i>B. typhosus</i> ... {	1	} No synergism.	—	—	—
	2		—	+	+
	3		+	+	+

The number of organisms present in one loop of the culture was ascertained by the pour-plate method. The results show that the synergistic mixtures are active only up to a certain stage and that if the organisms are abundant the bacteriostatic effect is not exerted. This, of course, is only to be expected in view of the fact that more drugs will be necessary to arrest growth of high bacterial population, since the changes which are achieved during the inhibitory processes are of the type of certain chemical reactions and a higher number of organisms normally need higher concentrations of the drugs.

Age of the culture.—Experiments were done with inocula from 24-, 48- and 72-hour old cultures. Growth was inhibited by penicillin alone and by penicillin-dye at the same concentration as for the younger organisms.

TABLE IX.

Effect of the age of culture on the synergistic concentrations of penicillin and dyes.

Organisms.	PENICILLIN + GENTIAN VIOLET.			PENICILLIN + BASIC FUCHSIN.		
	Age of culture in hours.					
	24	48	72	24	48	72
<i>B. dysenteriae</i> (Shiga) . . .	—	—	—	—	—	—
<i>B. typhosus</i> . . .	—	—	—	—	—	—

This study was conducted to see if there was any bearing on the age of the infection in *in vivo* condition with respect to the response to synergistic mixtures. The results, however, showed apparently no correlation with reference to its susceptibility to bacteriostatic mixtures. It may be pointed out that the *in vitro* tests cannot be of any help in this connection since one has to subculture the organisms young or old to fresh broth and then submit them to the action of bacteriostatic agents. If, on the other hand, the old cultures themselves are used in these experiments, there is the anomaly that the culture contains more organisms per c.c. than would be in a subculture and it has been seen in the previous table that the number of organisms indeed is a factor which determines susceptibility to bacteriostatic agents.

Morphological changes.—It was noted that where the suppression was only partial, the medium had a light granular turbidity but where suppression was fairly complete the medium was almost clear except for a slight granular deposit. When such cultures were centrifuged, stained and examined, it was found that the granular masses were composed largely of involution forms—such as long evenly staining filaments. When these cultures were, however, subcultured in nutrient broth

containing nucleic acid the normal morphology was regained, a finding in accord with our previous observation (George and Pandalai, 1948).

DISCUSSION.

A given bacterial cell can perform its essential living processes through two or more independent metabolic channels and can, therefore, keep on multiplying in the presence of a particular inhibitor effective against an important metabolic reaction. Different bacteriostatic agents are known to possess different bacteriostatic mechanisms and if these agents are allowed to function in conjunction with each other, there is a possibility of bringing about simultaneous inhibitory effects on more than one metabolic reaction pathways and consequently there may be an arrest of growth and multiplications of the organisms. There is a number of aspects which has to be explained with a view to have a clarified idea of the mechanism of the synergistic action. The theory that the synergic action of sulphonamides was due to their inhibition of penicillinase production, could be attributed in this case also, though the inhibition of penicillinase by the dyes has not the backing of published experimental evidence. Yet another theory can be suggested by the effects of inoculum size on the minimum bacteriostatic concentration of the penicillin. In the presence of the dye the number of test organisms in the inoculum will be reduced and bring them within the effective range of penicillin concentration which alone was unable to inhibit the original number or vice versa. The question of whether the drugs form complex compounds possessing enhanced bacteriostatic power also requires careful examination. Nevertheless, evidence is now available showing that a particular anti-bacterial agent, which is selective in its action on a particular variety of bacteria, can be made selective to other varieties also, provided that it is working in the presence of mixtures of bacteriostatic agents.

SUMMARY.

1. Synergic action of penicillin and bacteriostatic dyes, such as brilliant green, gentian violet, acriflavine, basic fuchsin, safranin and methylene blue, has been studied *in vitro* on the growth of Gram-negative bacilli like *B. dysenteriae* and *B. typhosus*.
2. Organisms which were inhibited to some extent by penicillin and the dye acting separately were inhibited under experimental conditions at lower concentrations when both agents acted together.
3. The effect of the synergistic mixtures on the size and age of the inoculum was also studied.
4. Certain theoretical aspects bearing on the mechanism of the synergic action have been discussed.
5. Further work to extend and elaborate the findings reported here and to assess the possibilities and limitations of the use of chemotherapeutic mixtures for clinical purposes in man is in progress.

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ANTISEPTICS OF THE ACRIDINE SERIES.

Part III.

BY

SURJIT SINGH, M.Sc. (Hons.),

J. R. CHAUDHRI, L.R.C.P. (Lond.), M.R.C.S. (Eng.), D.P.H. (Cantab.),

AND

MAHAN SINGH, M.Sc.

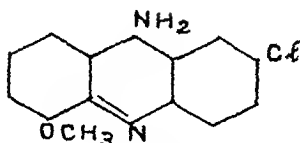
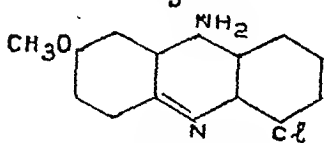
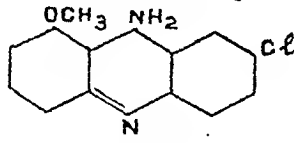
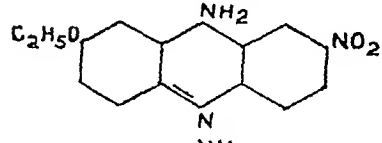
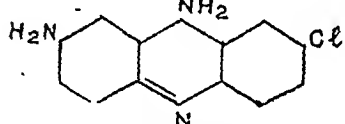
(From the East Punjab University, Simla.)

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IN previous communications (Surjit Singh and Chaudhri, 1947) we have reported the results relating to the antiseptic activity of a few derivatives of 3-methoxy-5-chloro-9-amino-acridine and 3-methoxy-7-(and 8)-chloro-9-amino-acridines with a view to finding any relation between antiseptic activity and chemical constitution. The conclusions arrived at are tentative and it is desirable that a number of other compounds of this series should be investigated before any definite conclusions could be drawn.

We have, therefore, undertaken the study of 38 more acridines belonging to five different series. The present paper is more in the nature of a survey and should serve as a guide for future synthetic work.

The compounds under investigation are derivatives of the following five acridines:—

1.  4-methoxy-7-chloro-9-amino-acridine.
2.  2-methoxy-5-(or 7)-chloro-9-amino-acridine.
3.  1-(or 3)-methoxy-7-chloro-9-amino-acridine.
4.  2-ethoxy-7-nitro-(or amino)-9-amino-acridine.
5.  2-amino-(or nitro)-7-chloro-(or bromo)-9-amino-acridine.

The same technique has been followed as was reported in the previous two communications (Surjit Singh and Chaudhri, 1947, 1948). The results are recorded in Tables I to V:—

TABLE I.

SERIES A. 4-methoxy-7-chloro-9-amino-acridine*.

Serial number.	Name.	Staph. aureus.		Bact. coli.	
		Bs.	Bc.	Bs.	Bc.
1	4-methoxy-7-chloro-9-amino-acridine ...	80	?	80	?
2	4-methoxy-7-chloro-9-(p'-toluidine)-acridine ...	10	<10	<10	<10
3	4-methoxy-7-chloro-9-(α-naphthyl)-amino-acridine	<10	<10	<10	<10
4	4-methoxy-7-chloro-9-(p'-anisidino)-acridine ...	<10	<10	10	10
5	4-methoxy-7-chloro-9-(p'-phenetidino)-acridine ...	40	<40	80	80

* For the synthetic part see Gurbakhsh Singh *et al.* (1947).

Note.—Bs.—denotes the effective bacteriostatic concentration of 1 in.....thousands.

Bc.—the bactericidal concentration 1 part in.....thousand part in this and subsequent tables.

TABLE I—concl'd.

SERIES A. 4-methoxy-7-chloro-9-amino-acridine*—concl'd.

Serial number.	Name.	<i>Staph. aureus.</i>		<i>Bact. coli.</i>	
		Bs.	Bc.	Bs.	Bc.
6	4-methoxy-7-chloro-9-(γ -diethyl-amino-propyl)-amino-acridine-2 HCl.	32	<32	8	<8
7	4-methoxy-7-chloro-9-(γ -dipropyl-amino-propyl)-amino-acridine-2 HCl.	16	<16	8	<8
8	4-methoxy-7-chloro-9-(γ -piperidino-propyl)-amino-acridine-2 HCl.	16	<8	64	<32
9	4-methoxy-7-chloro-9-(δ -diethyl-amino-butyl)-amino-acridine-2 HCl.	16	<8	16	<8
10	4-methoxy-7-chloro-9-(p'-sulphanilamido-phenyl)-amino-acridine.	<10	<10	<10	<10
11	4-methoxy-7-chloro-9-(p'-arseno-phenyl)-amino-acridine.	256	64	128	>32

* For the synthetic part see Gurbakhsh Singh *et al.* (1947).

Note.—Bs.—denotes the effective bacteriostatic concentration of 1 in.....thousands.

Bc.—the bactericidal concentration 1 part in.....thousand part in this and subsequent tables.

It would be observed that:—

- (1) the parent amino-acridine (No. 1) is fairly active. Its activity exceeds all compounds of this series excepting 4-methoxy-7-chloro-9-(p'-arseno-phenyl)-amino-acridine (No. 11). This is in accord with previous observations (Dupré and Robinson, 1945). Arsenic has long been used therapeutically for the cure of syphilis and chronic malaria. It was expected that a combination of an acridine with pentavalent arsenic should result in enhanced activity.
- (2) 4-methoxy-7-chloro-9-(p'-arseno-phenyl)-amino-acridine has also shown considerable activity against Shiga and Flexner dysentery with values (bactericidal) 20,000 and 40,000 respectively.
- (3) The sulphanilamide combination (No. 10) has yielded a product with poor activity. This supports the observation made by us before (*cf.* Part I, *loc. cit.*).
- (4) The groupings at position 9 do not follow any sequence. In Part I (*loc. cit.*) it was reported that the propyl group has the largest effect but it is not substantiated by the observations made in this series (*see* No. 7).
- (5) The aromatic closed ring substituents at position 9 which were expected to show very high activity have shown a poor activity (*see* Nos. 2, 3

and 4). The p'-phenetidino compound (No. 5) has, however, shown activity of the order of the parent compound.

- (6) When the chlorine atom is shifted from 7 to 6 keeping methoxy at 4, the activity is considerably increased. Thus, 4-methoxy-6-chloro-9-piperidino-acridine has (Bs. 160, Bc. 80) for *Staph. aureus* and (Bs. 160, Bc. 160) for *Bact. coli*.

TABLE II.

SERIES B. 2-methoxy-5-(or 7)-chloro-9-amino-acridine.

Serial number.	Name.	<i>Staph. aureus.</i>		<i>Bact. coli.</i>	
		Bs.	Bc.	Bs.	Bc.
1	2-methoxy-5-chloro-9-amino-acridine ...	10	<10	40	20
2	2-methoxy-5-chloro-9-(γ -diethyl-amino-propyl)-amino-acridine.	64	<32	32	<32
3	2-methoxy-5-chloro-9-(γ -dipropyl-amino-propyl-amino)-acridine.	8	4	16	<16
4	2-methoxy-5-chloro-9-(γ -piperidino-propyl)-amino-acridine.	4	2	4	4
5	2-methoxy-5-chloro-9-(δ -diethyl-amino-butyl)-amino-acridine.	128	8	32	32
6	2-methoxy-5-chloro-9-(p'-sulphanilamido-phenyl)-amino-acridine.	<10	<10	<10	<10

(1) The parent acridine 2-methoxy-5-chloro-9-amino-acridine is far less active than its isomer 3-methoxy-5-chloro-9-amino-acridine (Part I, *loc. cit.*). It would be seen that the shifting of the methoxy group from 3 to 2 position in this nucleus has brought about a dystherapeutic effect.

(2) When chlorine is shifted from position 5 to 7 the compound shows remarkable activity. The values for these compounds are given below for the sake of comparison. When, however, the 9-amino group is replaced by a chloro group in the above compound, the resultant compound is inactive.

Serial number.	Name.	<i>Staph. aureus.</i>		<i>Bact. coli.</i>	
		Bs.	Bc.	Bs.	Bc.
1	2-methoxy-5-chloro-9-amino-acridine ...	10	<10	40	20
2	2-methoxy-7-chloro-9-amino-acridine ...	320	<320	640	<640
3	2-methoxy-7-chloro-9-chloro-acridine ...	<2	<2	<2	<2

(3) The sulphanilamido derivative (No. 6) is inactive as anticipated from our previous observations.

(4) 2-methoxy-7-chloro-9-(p'-arseno-phenyl)-amino-acridine which is fairly active against *Staph. aureus* (Bs. 128, Bc. 64) and *Bact. coli* (Bs. 256, Bc. 128) has, however, comparatively feeble action against dysentery organisms, the values being 10,000 against Shiga and Flexner.

TABLE III.

SERIES C. 1-(or 3)-methoxy-7-chloro-9-amino-acridine.

Serial number.	Name.	<i>Staph. aureus.</i>		<i>Bact. coli.</i>	
		Bs.	Bc.	Bs.	Bc.
1	1/3-methoxy-7-chloro-9-(δ -diethyl-amino-butyl-amino)-acridine.	16	16	256	64
2	1/3-methoxy-7-chloro-9-(γ -diethyl-amino-propyl)-amino-acridine.	16	16	128	128
3	1/3-methoxy-7-chloro-9-(γ -dipropyl-amino-propyl)-amino-acridine.	16	16	64	<64
4	1/3-methoxy-7-chloro-9-(γ -piperidino-propyl)-amino-acridine.	16	16	32	32

In the above series it has not been possible to find out whether the ring closure leads to the formation of 1-methoxy or 3-methoxy compound. It is, therefore, not possible to draw any conclusion in regard to the position of the methoxy group.

The compounds noted above are, however, quite active. They have uniformly the same activity against *Staph. aureus*, while the values for *Bact. coli* are widely divergent.

TABLE IV.

SERIES D. 2-ethoxy-7-nitro-(or amino)-9-amino-acridine.

Serial number.	Name.	<i>Staph. aureus.</i>		<i>Bact. coli.</i>	
		Bs.	Bc.	Bs.	Bc.
1	2-ethoxy-7-amino-9-(p'-chloro-anilino)-acridine ...	80	<10	<10	<10
2	2-ethoxy-7-amino-9-(p'-phenetidino)-acridine ...	640	<640	640	<640
3	2-ethoxy-7-(p'-acetyl-amino-phenyl-sulphonyl)-amino-9-(p'-bromo-anilino)-acridine.	<10	<10	<10	<10
4	2-ethoxy-7-nitro-9-amino-acridine ...	40	40	160	<160
5	2-ethoxy-7-nitro-9-(p'-arseno-phenyl)-amino-acridine.	256	64	256	64

(1) 2-ethoxy-7-amino-9-(p'-phenetidino)-acridine (No. 2) has shown remarkable activity. It is in fact the most active of all the 38 compounds described in this paper.

(2) The sulphanilamide derivative (No. 3) is inactive as was expected. The extreme inactivity of this compound may be partly due to the p'-bromo atom which is also the case with p'-chloro-anilino compound (No. 1).

(3) The change of 7-amino to 7-nitro does not result in any appreciable decrease in the activity (see Nos. 4 and 5). Albert *et al.* (1945) have recommended 3-nitro-9-amino-acridine due to its very negative reduction potential and ionic resonance. Nitro group does not seem to have any dystherapeutic effect.

(4) The arsenic acid derivative has shown high activity against *Staph. aureus* and *Bact. coli* and has shown great specificity for Flexner dysentery for which its effective bactericidal concentration is 1 : 320,000, while against Shiga it is very low, 1 : 10,000 only.

TABLE V.

SERIES E. 2-amino-(or nitro)-7-chloro-(or bromo)-9-amino-acridine.

Serial number.	Name.	<i>Staph. aureus.</i>		<i>Bact. coli.</i>	
		Bs.	Bc.	Bs.	Bc.
1	2-amino-7-bromo-9-anilino-acridine ...	<10	<10	<10	<10
2	2-amino-7-bromo-9-(p'-anisidino)-acridine ...	20	10	320	160
3	2-amino-7-bromo-9-(p'-bromo-anilino)-acridine ...	<10	<10	<10	<10
4	2-amino-7-chloro-9-(p'-toluidino)-acridine ...	320	<320	640	<640
5	2-acetylamino-7-chloro-9-(p'-anisidino)-acridine ...	<2	<2	<2	<2
6	2-(acetylamino-phenyl-sulphanilamide)-7-chloro-9-(p'-toluidino)-acridine.	<2	<2	<2	<2
7	2-nitro-7-bromo-9-amino-acridine ...	20	10	20	20
8	2-nitro-7-chloro-9-(p'-chloro-anilino)-acridine ...	10	<10	640	<640

It would be observed that in presence of amino group at position 2, a halogen atom leads to the decrease in the activity of the compound. The effect of these compounds is, however, partly neutralized by the p'-toluidino group and

p'-anisidino group at the 9 position. The bromo derivatives are more inert than the chloro ones.

When, however, the amino group is acetylated (see No. 5) the fall in the activity is phenomenal. A similar fall in the activity would be noticed when the sulphanilamide grouping is introduced (see No. 6). It is thus clear that the substitution of 2-amino group is altogether undesirable.

When the amino group is changed for nitro, the activity of the compound is reduced (see Nos. 7 and 8) but not completely annulled in spite of the presence of halogen at 7.

SUMMARY.

1. Most of the compounds tested have shown marked bactericidal activity against *Bact. coli* and *Staph. aureus*. This is particularly true of 2-ethoxy-7-amino-9-phenetidino-acridine; 2-methoxy-7-chloro-9-(p'-arsino-phenyl)-amino-acridine and 2-amino-7-chloro-9-(p'-toluidino-acridine). Some compounds have shown high specificity against *Bact. coli* only, for instance 2-nitro-7-chloro-8-p'-chloro-anilino-acridine and 2-amino-7-bromo-9-phenetidino-acridine. In almost all cases, however, bactericidal activity has been greater against *Bact. coli* than against *Staph. aureus*.

2. Sulphonamide combination with acridine (or its variations

$\text{-NH} \text{---} \text{C}_6\text{H}_4 \text{---} \text{SO}_2\text{NH}_2$ or $\text{-HNSO}_2 \text{---} \text{C}_6\text{H}_4 \text{---} \text{NHCOCH}_3$ —whether at position

7 or 9 in the acridine nucleus) makes the compound inert. This was expected from our previous observations (cf. Part I, *loc. cit.*) and those of Lawrence (1943) who has also described similar results with 3-chloro-7-methoxy-9-(4'-sulphamido-benzyl)-amino-acridine.

3. Halogens at position 9 make the compounds inactive. Bromo compounds are particularly so, whether at position 7 or 9, whereas 7-chloro derivatives are not so inert.

4. Arsenic acid (pentavalent arsenic) derivatives are quite effective against *Bact. coli* and *Staph. aureus*, while in some cases against Shiga and Flexner as well.

5. Relative position of substituents is as important as the substituents themselves. Minor nuclear change produces great difference in the activity. Thus, unsubstituted amino group at position 9 or 2 is most desirable, any tempering (even acetylation) leads to a dystherapeutic effect. The substituents in order of their activity are—*aniline*, *toluidine*, *anisidine*, *piperidine*, *phenetidine*, etc.

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A SIMPLE METHOD FOR THE REMOVAL OF INTERFERING SUBSTANCES IN THE ESTIMATION OF THIAMINE IN URINE.

BY

R. MUKUNDAN.

AND

B. V. RAMA SASTRI.

(From the Nutrition Research Laboratories, Indian Research Fund Association, Coonoor.)

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It is generally agreed that the main difficulty in the determination of thiamine in urine is the interference due to the presence of isobutanol-extractable fluorescent impurities. Various workers have tried to overcome this difficulty in different ways. Wang and Harris (1939) used preliminary washing of urine with isobutanol. Mason and Williams (1942) used a blank in which the thiamine had been destroyed by heating with sulphite. Wang and Harris (1943) used hydrogen peroxide after oxidation of thiamine. Najjar and Ketron (1944) introduced a correction factor for the reduction of fluorescence caused by the oxidation of the niacin derivative F_2 occurring normally in urine. Mickelson, Condiff and Keys (1945) do not consider any of the above methods as satisfactorily eliminating the interference caused by the variability of the blank. They used a mixture of hydrochloric acid and phosphoric acid to adjust to pH 8 to 9.5 before the final extraction with isobutanol. In an investigation on the thiamine requirements of Indian adults, it was found that in the above methods there was no possibility of eliminating completely the colour of urine (especially highly coloured urine) to minimize the interference of the urinary pigments and to get a better recovery of the added thiamine.

A simple modification of the accepted thiochrome method is suggested whereby the interfering pigments can be effectively eliminated. Bhagvat (1943) observed that treatment of the enzymic extract of the foodstuff with basic lead acetate, instead of preliminary washing, removed effectively the substances which interfered with the estimation of thiamine. This observation has been applied for the removal of interfering substances in the determination of thiamine in urine. The modified procedure is as follows :—

To 40 c.c. of urine in a flask are added about 20 c.c. to 25 c.c. of N basic lead-acetate solution. A bulky precipitate forms; the completion of the precipitation is tested by a further addition of 2 to 3 drops of basic lead-acetate solution to the

clear liquid after allowing the precipitate to settle down. The precipitate is filtered off and washed 2 to 3 times using 5 c.c. of water for each washing. The excess lead in the filtrate is removed as lead sulphate by the addition of 1 c.c. to 2 c.c. of 10 N sulphuric acid. The lead sulphate is filtered and washed 2 to 3 times with 5 c.c. portions of distilled water and the combined filtrate made up to 100 c.c. Aliquots of the solution representing 4 c.c. to 8 c.c. of urine (according to the expected thiamine content) are taken for further treatment according to the method of Wang and Harris (1943). Low blanks and good recoveries are obtained by adopting this procedure. It is particularly useful when highly coloured urines are concerned.

In Table I are given the figures for thiamine excretions of normal individuals as estimated by the method of Wang and Harris (1943) without basic lead-acetate treatment. Table II contains results obtained by the modified method and in Table III is given the comparison of the two methods using the same urine :—

TABLE I.

Subject.	Urine excreted in 24 hours, c.c.	Thiamine excretion, $\mu\text{g.}/24$ hours.	Recovery percentage of added thiamine.
K	2,120	106	89
T	2,209	196	69
K	2,240	75	77
T	2,340	106	50
T	1,910	73	62
P	1,570	505	67

TABLE II.

Subject.	Urine excretion in 24 hours, c.c.	Thiamine excretion, $\mu\text{g.}/24$ hours.	Recovery percentage of added thiamine.
K	2,145	204	100
T	2,165	109	95
P.G.	2,005	251	89
R	2,340	426	100
N	2,030	254	107
P.G.	3,080	560	92

TABLE III.

Subject.	Urine excretion in 24 hours, c.c.	WITHOUT BASIC LEAD-ACETATE TREATMENT.		WITH BASIC LEAD-ACETATE TREATMENT.	
		Thiamine excretion, μ g./24 hours.	Recovery percentage.	Thiamine excretion, μ g./24 hours.	Recovery percentage.
R	1,920	101	75	137	93
S	2,380	207	76	486	93
K	2,620	174	94	175	107
H	2,510	433	70	570	100
K	2,520	84	85	168	90
*K	2,030	†	...	110	88

* After administration of 100 mg. of nicotin amide.

† Blank is higher than after oxidation with ferricyanide.

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IMMUNO-CHEMICAL STUDIES OF *VIBRIO CHOLERÆ*. A PRELIMINARY NOTE.

BY

D. L. SHRIVASTAVA,
GURKIRPAL SINGH,

AND

M. L. AHUJA.

(From the Cholera Inquiry under the Indian Research Fund Association
at the Central Research Institute, Kasauli.)

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THE classical discovery of the specific polysaccharides of *pneumococci* by Avery and Heidelberger (1923, 1925) gave concrete direction to the importance of immuno-chemical studies of micro-organisms. MacLeod *et al.* (1945) and Heidelberger *et al.* (1946) have recently reported on the successful immunization of human subjects with specific polysaccharides of *pneumococcus*.

Landsteiner and Levine (1927) extracted a substance having the characteristics of a polysaccharide from a vibrio strain. Linton (1940) isolated polysaccharides from various strains of vibrios but their earlier preparations were serologically inactive. Later in their work Shrivastava and Seal (1937) prepared a vibrio polysaccharide which was active serologically but failed to produce antibodies in rabbits. White (1937), whose studies have supplied so far the most valuable information on vibrio polysaccharides, succeeded in producing antibodies in rabbits by injecting 'protein-free' specific substance.

The observations recorded below give results of protection experiments using polysaccharides isolated from *V. cholerae* as active immunizing agents.

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